INTERNATION SEARCH REPORT

Intr Idio Application No
PCT/US 97/20014

PC1/US 9//	2001
CLASSIFICATION OF SUBJECT MATTER PC 6 C07H21/00 C1201/68 C07H23/00	
coording to International Patent Classification (IPC) or to both national classification and IPC	
GIELDS SEARCHED	
inimum documentation searched (classification system followed by classification symbols) PC 6 C07H C12Q	
ocumentation searched other than minimumdocumentation to the extent that such documents are included in the fields sea	arched
iectronic data base consulted during the international search (name of data base and, where practical, search terms used	
C. DOCUMENTS CONSIDERED TO BE RELEVANT	
Catagory Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
WO 95 15971 A (CALIFORNIA INST OF TECHN) 15 June 1995 cited in the application	1-29
see claims 1-20: figures 1-4: examples 1-7 R.P.HSUNG ET AL.: "Synthesis and CHaracterization of Unsymmetric Ferrocene-Terminated Phenylethynyl Oligomers."	1-29
ORGANOMETALLICS, vol. 14. no. 10. 1995. pages 4808-4815, XP002077968 cited in the application see the whole document	
WO 93 10267 A (IGEN INC) 27 May 1993 see abstract; claim 1 -/	1-29
Further documents are listed in the continuation of box C	ed in annex.
Special categories of cited documents	theory underlying the sectained invention into the considered to document is taken alone the claimed invention in inventive step when the impre other such docuvious to a person skilled entitating the control of the such docuvious to a person skilled entitlement.
Date of the actual completion of theinternational search 18 September 1998 - 01/10/1998	search report
Name and mailing address of the ISA Authorized officer	

European Patient Office, P.B. 5818 Patentiaan 2 IIL - 280 HV Rijswijk

INTER ATIONAL SEARCH REPORT

Inte Ional Application No PCT/US 97/20014

		PC1/US 9//20014			
(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT					
Category :	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No			
Janagory					
1	WO 90 05732 A (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 31 May 1990	1-29			
	see the whole document				
Y	WO 94 22889 A (CIS BIO INT ; TEOULE ROBERT (FR): ROGET ANDRE (FR): LIVACHE THIERRY) 13 October 1994	1-29			
	see abstract; claims 13-18; examples 6-8				
Υ	TURRO N J ET AL: "PHOTOELECTRON TRANSFER BETWEEN MOLECULES ADSORBED IN RESTICTED SPACES" PROCEEDINGS OF THE INTERNATIONAL	1-29			
	CONFERENCE ON PHOTOCHEMICAL CONVERSION AND STORAGE OF SOLAR ENERGY, 1990, pages 121-139, XP002041921 see the whole document				
Y	MURPHY C J ET AL: "LONG-RANGE PHOTOINDUCED ELECTRON TRANSFER THROUGH A DNA HELIX" SCIENCE.	1-29			
	vol. 262, 12 November 1993, pages 1025-1029, XP002041920 see the whole document				
A	AIZAWA M ET AL: "INTEGRATED MOLECULAR SYSTEMS FOR BIOSENSORS*" SENSORS AND ACTUATORS B. vol. B24. no. 1/03. PART 01. March 1995. pages 1-5. XP000521315 see abstract	1.9			
А	J.R.REIMERS ET AL.: "Towards Efficient Molecular Wires and Switches: The Brooker Ions." BIOSYSTEMS. vol. 35, 1995, pages 107-111, XP002077969 see the whole document	1.2.9			
P.Y	WO 96 40712 A (CALIFORNIA INST OF TECHN) 19 December 1996 see claims 1-27: examples 1-8	1-29			
P,Y	W.M.ALBERS ET AL.: "Design of Novel Molecular Wires for Realizing Long-Distance Electron Transfer." BIOCHEMISTRY AND BIOENERGETICS. vol. 42, 1997, page 25-33 XP002077970 see the whole document	1-29			
P.Y	US 5 591 578 A (T.J.MEADE ET AL.) 7 January 1997 see the whole document	1-29			

INTER TONAL SEARCH REPORT

Information on patent family members

int ional Application No PCT/US 97/20014

 Patent document cited in search report		Publication date		ent family emberis)	Publication date
WO 9515971	A	15-06-1995	US AU CA EP JP US US	5591578 A 1215295 A 2178618 A 0733058 A 9506510 T 5770369 A 5780234 A 5705348 A	07-01-1997 27-06-1995 15-06-1995 25-09-1996 30-06-1997 23-06-1998 14-07-1998 06-01-1998
WO 9310267	Α	27-05-1993	AU AU CA EP IL JP JP US ZA	658962 B 3141293 A 2100159 A 0567635 A 103754 A 2788786 B 6507316 T 5635347 A 9208839 A	04-05-1995 15-06-1993 16-05-1993 03-11-1993 15-04-1997 20-08-1998 25-08-1994 03-06-1997 13-05-1993
WO 9005732		31-05-1990	US AU CA US	5112974 A 4647689 A 2002380 A 5439794 A	12-05-1992 12-06-1990 07-05-1990 08-08-1995
WO 9422889	A	13-10-1994	FR AT DE DE DK EP ES GR JP	2703359 A 159028 T 69406119 D 69406119 T 691978 T 0691978 A 2110228 T 3025738 T 8508311 T	07-10-1994 15-10-1997 13-11-1997 26-03-1998 25-05-1998 17-01-1996 01-02-1998 31-03-1998 03-09-1996
WO 9640712	A	19-12-1996	AU US	6166296 A 5770369 A	30-12-1996 23-06-1998
us 5591578	 А	07-01-1997	AU CA EP	1215295 A 2178618 A 0733058 A	27-06-1995 15-06-1995 25-09-1996

INTERNATIONAL SEARCH REPORT

Interior No PCT/US 97/20014

	II (I LERI (I LE)	PCT/US 97/20014			
.(Continua	NION) DOCUMENTS CONSIDERED TO BE RELEVANT	Relevant to claim No			
atedota	Citation of document, with indication,where appropriate, of the relevant passages		7,573		
	P.LINCOLN ET AL.: "Short-Circuiting the Molecular Wire." JOURNAL OF THE AMERICAN CHEMICAL SOCIETY vol. 119, no. 6, 1997, pages 1454-1455, XP002077971 DC US see the whole document		1.9		

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07H 21/00, C12Q 1/68 C07H 23/00	А3	 (11) International Publication Number: WO 98/20162 (43) International Publication Date: 14 May 1998 (14.05.98)
(21) International Application Number: PCT/US9 (22) International Filing Date: 5 November 1997 (0)		Albritton & Herbet LLP, Suite 3400, 4 Embarcadero Center,
(30) Priority Data: 08/743,798 5 November 1996 (05.11.96) 60/040,155 7 March 1997 (07.03.97) 08/873,597 12 June 1997 (12.06.97) 08/873,978 12 June 1997 (12.06.97) 08/899,510 24 July 1997 (24.07.97) 08/911.085 14 August 1997 (14.08.97) 08/911.589 14 August 1997 (14.08.97) (71) Applicant (for all designated States except US): CL MICRO SENSORS [US/US]; 101 Waverly Drive, P CA 91105 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KAYYEM, [US/US]; 428 South Sierra Bonita Avenue, Pased 91106 (US). O'CONNOR, Stephen, D. [US/US) South El Molino #16, Pasadena, CA 91106 (US). Michael [IL/US]; 276 South El Molino #33, Pas: 91101 (US). YU, Changjun [CN/US]; 400 Ray Drive #32, Pasadena, CA 91030 (US).	Jon, lena COZI COZI dera, C	Published With international search report. Before the expiration of the time limit for amending the claim. and to be republished in the event of the receipt of amendments. No. (88) Date of publication of the international search report:

(57) Abstract

The invention relates to nucleic acids covalently coupled to electrodes via conductive oligomers. More particularly, the invention is directed to the site-selective modification of nucleic acids with electron transfer moieties and electrodes to produce a new class of biomaterials, and to methods of making and using them.



Information on patent family members

Ì		Application No
	PCT/US	97/20014

		\ ' ' ' _	
Patent document	Publication date	Patent family member(s)	Publication date
US 5591578 A	dan	JP 9506510 T WO 9515971 A US 5770369 A US 5780234 A US 5705348 A	30-06-1997 15-06-1995 23-06-1998 14-07-1998 06-01-1998

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL.	Afbania	ES	Spain	LS	Lesotho	SI	Slovenia
	Amenia	FI	Finland	LT	Lithuania	SK	Slovakia
AM AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AU AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MĐ	Republic of Moldova	TG	Togo
	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BB	Belgium Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BE BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
	***	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BG	Bulgaria	IE.	Ireland	MN	Mongolia	UA	Ukraine
BJ	Benin Brazil	11.	Israel	MR	Mauritania	UG	Uganda
BR		IS	leeland	MW	Malawi	US	United States of America
BY	Belarus	IT	Italy	MX	Mexico	UZ	Uzbekistan
CA	Canada	JP	Japan	NE	Niger	VN	Viet Nam
CF	Central African Republic	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CG	Congo	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CH	Switzerland	KP	Democratic People's	NZ	New Zealand		
CI	Côte d'Ivoire	Kt.	Republic of Korea	PL.	Poland		
CM	Cameroon	r n	Republic of Korea	PT	Portugal		
CN	China	KR	Kazakstan	RO	Romania		-
CU	Cuba	KZ LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic		Liechtenstein	SD	Sudan		
DE	Germany	Ll		SE	Sweden		
DK	Denmark	1.K	Sri Lanka	SG.	Singapore		
EE	Estonia	1.R	Liberia	30	Singapore		

The use of an alternate protecting group for protection of the sulfur atom prior to attachment to the gold surface was explored.

To 0.5 gm of molecular sieve (3 Å) was added 3 ml of dry THF and 2.5 ml of 1.0 tetrabutylammonium fluoride. After stirring for 20 minutes, 100 mg of compound #1 was added under Argon. The reaction mixture was stirred for 1 hour and poured into 100 ml of 5% citric acid solution and the aqueous solution was shaken well and extracted twice with either (2 X 100 ml). The combined ether solution was dried over Na₂SO₄ and concentrated. The residue was purified by column chromatography using 10% CH₂Cl₂/Hexane as eluent. The purified product was analyzed by ¹HNMR which should 50% of compound #2 and 50% of the corresponding disulfide.

The use of this protecting group in synthesizing base-attached conductive oligomers is depicted in Figures 20 and 21.

20

25

30

35

15

5

10

Example 12

Preparation of Peptide Nucleic Acids with Electron Transfer Moieties

The synthesis of a peptide nucleic acid monomeric subunit with a conductive oligomer covalently attached to the α-carbon is depicted in Figure 31.

4-lodophenylalanine: Into a solution of 40.15 gm (0.243 mol) of phenylalanine in a mixture of 220 mL of acetic acid and 29 mL of concentrated H₂SO₄ was added 24.65 gm (0.097 mol) of powered iodine and 10. 18 gm(0.051 mol) of powered NalO₃ while stirring. The reaction mixture was stirred at 70 °C for 21 h, during this time, two portions of 1 gm of NalO3 were added. The mixture was cooled and the acetic acid was removed by using rotavapor while temperature was maintained at 35°C and the residue oil was diluted by adding 400 mL of water. The aqueous solution was extracted once with 100 mL ether and once with 100 of dichloromethane. After decolorization with 5 gm of Norit, the aqueous solution was neutralized by adding solid NaOH to precipitate the crude product, which, after chilling, was filtered and rinsed with 800 mL of water and 300 mL of ethanol. The wet product was recrystallized from 200 mL of acetic acid to produce 37.5 gm of 4-iodo-L-phenylalanine.

5

10

15

20

25

Methyl 4-lodophenyl Alaninate Hydrochloride: To 10 mL of methanol cooled in an ice-water bath was added dropwise 10.2 gm of thionyl chloride. Into the cold solution was added 5.0 gm of 4-iodophenylalanine and the yellow solution was formed and refluxed for 2 h. After removing the solvent, the white solid was obtained and recrystallized from 10 mL of methanol by addition of 50 mL of ether. The title product(5.4 gm) was prepared.

Methyl N-Amidocarboxylethyl-4-lodophenyl Alaninate: To a solution of 5.0 gm (14.6 mmol) of methyl 4-iodophenylalaninate hydrochloride in 100 mL of acetonitrile was added 6 mL of triethylamine and 1.1 gm (15.4 mmol) of acrylamide. The solution was stirred overnight. After removing the solvent, the residue was dissolved in 200 mL of dichloromethane and the solution was washed once with 5 % $NaHCO_3$ solution and dried over sodium sulfate. The product was purified by column separation.

Methyl N-Aminoethyl-4-lodophenyl Alaninate: To a solution of 3.46 gm (8 mmol) of [I,I-bis(trifloroacetoxy)iodo]benzene in 24 mL of acetonitrile was added 12 mL of the glass-distilled water, followed adding 2.98 gm(8 mmol) of methyl N-amidocarboxylethyl alninate. The mixture was stirred for 6 h at room temperature and diluted by 150 mL of water and 16 mL of concentrated HCl solution. The aqueous solution was extracted once with 150 mL of ether and concentrated to about one third of the original volume. The concentrated NaOH solution was used to adjust pH of the aqueous solution to greater than 12 and the basic water solution was extracted 6 times with CH₂Cl₂ (6 x 200 mL). The combined extracts were dried over anhydrous sodium sulfate and concentrated to dryness and further dried over a high vacuum line and the product was used for next step without further purification.

Methyl N-(2-Nitrobenzenesulfonyl)-4-lodophenyl Alaninate: To a two-necked flash was added 19.0 g (55.8 mmol) of methyl N-aminoethyl-4-iodophenyl alaninate and 600 mL of dry DMF. The resulting solution was cooled in an ice-water bath. Into the cold solution was added 20 mL of TEA, followed by adding 13.5 gm (60.9 mmol) of 2-nitrobenzenesulfonyl chloride portion by portion. The mixture was stirred at low temperature for 30 min. and warmed up to room temperature and stirred for another 4 h. The precipitate was formed and filtered off and washed once with DMF. After removing DMF on the high vacuum, the residue was dissolved in the 500 mL of dichloromethane. The organic solution was washed twice with the brine and dried ver Na₂SO₄ and then concentrated. The residue was dissolved in a small amount of dichloromethane for the column purification. Silica gel (250 gm) was packed with CH₂CL₂, the sample solution was loaded and the column was eluted with CH₂CL₂. The fractions were identified by TLC (CH₂Cl₂ as developing solvent) and pooled and concentrated to afford 24.1 gm (88.1%) of the title compound

35

5

10

15

20

25

30

35

Methyl N-(2-MMT-aminoethyl)-N-(2-Nitrobenzenesulfonyl)-4-lodophenyl Alaninate: To a solution of 16.5 gm (49.5 mmol) of 2-MMT-amino ethanol, 20.0 gm (40.8 mmol) of methyl N-(2-nitrobenzensulfonyl)-4-iodophenyl alaninate and 13 gm (49.5 mmol) of triphenylphosphine in 250 mL of dry THF cooled in an ice-water bath was added 7.8 mL (49.5 mmol) of diethyl azodicarboxylate under Argon. The solution was warmed up to room temperature and stirred overnight. After removing THF, the residue dissolved in the small amount of the CH₂Cl₂ for column separation. TLC (CH₂Cl₂: Hexane = 9:1) of the sample solution indicated two products, i.e., the early spot is the desired product, the later spot is triphenylphosphine oxide. Silica gel (300 gm) was packed with 1% TEA/hexane. The sample solution was loaded and the column was eluted with 500 mL of 1% TEA/hexane, 100 mL of 1% TEA/25% CH₂Cl₂/hexane and 1000 mL of 1% TEA/50% CH₂Cl₂/hexane. The fractions were identified by TLC (CH₂Cl₂:Hexane = 9:1). The fractions containing the pure early spot were pooled and concentrated to give 17 gm of the title compound. The overlapping fractions were pooled, concentrated and repurified to give another 3.0 gm of the title compound. The total yield is 62.0%.

Methyl N-(2-MMT-aminoethyl)-4-iodophenyl Alaninate: To a suspension of 17.0 gm (21 mmol) of methyl N-(2-MMT-aminoethyl)-N-(2nitrobenzenesulfonyl)-4-iodophenyl alaninate, 11.6 gm (84 mmol) of Potassium Carbonate in 150 mL of DMF was added 2.6 mL (25.8 mmol) of thiophenol under Argon. The reaction mixture was stirred at room temperature for 1.3 h. and diluted by adding 1.2 L of the brine. The aqueous solution was extracted three times by ether (2x 500 mL) and the combined extracts was washed once with the diluted NaOH solution and dried over sodium sulfate. After the removal of the solvent, the residue was used for column separation. Silica Gel (220 gm) was packed with 1% TEA/hexane, upon loading the sample solution, the column was eluted with 500 mL of 1% TEA/hexane. 1000 mL of 1% TEA/25% ether/hexane, and 1000 mL of 1% TEA/50% ether/hexane. The fractions were identified by TLC (Ether:Hexane) and pooled and concentrated to afford 5.6 gm (43.1%) of the title product.

Methyl N-(2-MMT-aminoethyl)-N-[(Thymin-1-yl)acetyl]-4-lodophenyl Alaninate: To a solution of 3.37 g (5.43 mmol) of methyl N-(2-MMT-aminoethyl)-4-iodophenyl alaninate in DMF (10 mL) was added 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (.884 g, 5.43 mmol) and 4-e/hylmorpholine (1.38 mL, 10.86 mmol). A solution of thymine acetic acid (1.00 g, 5.43 mmol) in DMF (10 mL) was then added, followed by N,N'-diisopropylcarbodiimide (1 mL, 6.5 mmol). The reaction mixture was left stirring overnight at room temperature for 20.5 h. The solvent was removed in vacuo. The residue was dissolved in 600 mL of CH₂Cl₂ and the solution was washed with twice with 500 mL of water and once with 500 mL of brine and dried in Na₂SO₄. After the removal of the solvent, the crude residue was dissolved in ~10mL of CH₂Cl₂ for column separation. Silica gel (135 gm) was packed with 1% TEA/CH₂Cl₂. The

fractions were identified by TLC ($CH_2Cl_2:CH_3OH=95:5$). The fractions containing the desired product were pooled and concentrated to dryness. The solid product was dissolved in minimum amount of EtOAc and left in the freezer to precipitate out n,n'diisopropylurea. The precipitate was filtered and the filtrate was concentrated to afford 3.56 g (83.3%) of the title compound.

5

10

15

N-(2-MMT-aminoethyl)N-[(Thymin-1-yl)acetyl]-4-lodophenyl Alaninate: 3.5 g (4.45 mmol) of methyl N-(2-MMT-aminoethyl)-N[(thymin-1-yl)acetyl]-4-iodophhenyl alaninate was dissolved in dioxane (20 mL) and water (4 mL). The solution was cooled to 0°C and 1 M NaOH was added dropwise until the pH=12. After 1 h., the reaction mixture was warmed to room temperature and more 1 M NaOH was added and the pH remained at 12. The reaction was monitored by TLC (CH₂Cl₂:CH₃OH=95:5). After the hydrolysis was complete, the pH of the reaction mixture was adjusted to 5 with 2 M KHSO₄. Then it was diluted by adding 300 mL of CH₂Cl₂. The organic layer was separated and the aqueous layer was extracted twice with 250 mL of CH₂Cl₂. The combined organic extracts were dried over Na2SO4 and concentrated. The residue was dissolved in minimum amount of CH₂Cl₂ for column purification. Silica gel (52 gm) was packed with 1% TEA/2% CH₃OH/CH₂Cl₂, after loading the sample, the column was elute with 700 mL of 1% TEA/2%CH₃OH/CH₂Cl₂ and 1 L of 1% TEA/5% CH₃OH/CH₂Cl₂. The fractions were identified by TLC (CH₂Cl₂:CH₃OH=95:5). The removal of the fractions containing the desired product gave 2.9 g(84.6%) of the title compound.

20

25

PNA-Backbone-Wire: A mixture of 1g (1.29 mmol) of N-(2-MMT-aminoethyl)-N-[(thymin-1-yl)acetyl]-4-iodophenyl alaninate, 0.5 g (1.29 mmol) of trimethyl silyl ethyl protected 3-unit wire, 44.6 mg (0.077 mmol) of Pd(dba)₂, 91.6 mg (0.349 mmol) of triphenylphosphine, and 44.6 mg (0.17 mmol) of copper (I) iodide 120 mL of DMF and 62 mL of pyrrolidine was degassed well and stirred at 60°C for 5h. The solvent was removed and the residue was dissolved in 250 mL of CH₂Cl₂ and 200 mL of saturated EDTA solution. This mixture was stirred for 30 min. The organic layer was separated,, dried over sodium sulfate and concentrated. The crude product was dissolved in minimum CH₂Cl₂ for column separation. Silica gel (22 gm) was packed with 1% TEA/CH₂Cl₂, upon loading the sample solution, the column was eluted with 1L of 1% TEA/2%CH₃OH/CH₂Cl₂ and 1% TEA/5% CH₃OH/CH₂Cl₂ until finishing the separation. The fractions were identified by TLC (CH₂Cl₂·CH₃OH=95:5). The right fractions were combined and concentrated to afford 0.55 g of yellow-orange solid, which was dissolved in 150 mL of CH₂Cl₂ and diluted by adding 50 mL of water and 50 mL of 10% tetrabutylamine hydroxide. The mixture was placed in a separatory funnel and shaken for 5 min. The organic layer was separated and the aqueous layer was extracted once more with 50 mL of CH₂Cl₂ and the combined organic layer was dried in Na₂SO₄. The solvent was removed to afford 0.8 g (46.5%

35

of the title product.

5

10

15

20

25

30

35

-93-

Example 13

Preparation of Peptide Nucleic Acids with Electron Transfer Moieties

The synthesis of a peptide nucleic acid monomeric subunit with a ferrocene electron transfer moiety covalently attached to the base is depicted in Figure 32.

Synthesis of Y1: 5-lodo uracil (100.0 gm) was suspended in 250 ml of dry DMF. 1.68 gms of sodium hydride was added in portions. The reaction mixture was then stirred at room temperature for 40 minutes. Then 6.16 ml of t-butyl bromoacetate ws added and the reaction mixture was stirred for an additional two hours at room temperature. The reaction mixture was quenched with 5 ml of methanol containing CO². The solvent was then removed and the residue was dissolved in dichloromethane and washed with water. The precipitate was formed during the wash and then filtered and dried. The reaction yielded 9.33 g of product Y1.

Synthesis of Y2: To a solution of 6.33 g of Y1 in 140 ml of dichloromethane was added 35 ml of triethylamine, 0.55 g of 4-dimethylaminopyridine, and 5.89 g of 2-mesitylenesulfonyl chloride. The reaction mixture was stirred for 40 minutes and then 0.40 g of 1.4-diazobicyclo[2,2,2] octane and 4.34 ml of 2,4-dimethylphenol were added and stirred for 2 hours. The reaction mixture was then diluted by adding 200 ml of dichloromethane and the solution was washed with a 5% sodium bicarbonate solution, dried over sodium sulfate and concentrated. The residue was dissolved in 5 ml of dichloromethane and loaded onto a 200 g silica gel column packed with dichloromethane. The column was eluted with 1-5% methanol/dichloromethane. The fractions containing the diesired product was pooled and concentrated to give 2.5 g of Y2.

Synthesis of Y3: A mixture of 2.5 g of Y2, 1.38 g of ferrocene acetylene, 200 mg of Pd(pph₃)Cl₂ and 208 mg of copper iodide in 100 ml of dimehtylformamide (DMF) and 100 ml of triethylamine was degassed well and stirred at 55°C for 2 hours. Upon removing solvent the residue was dissolved in dichloromethane and the solution was washed with a 5% sodium bicarbonate solution, dried over sodium sulfate and concentrated. The crude residue was dissolved in 5 ml of dichloromethane and loaded onto a 200 g silica gel column packed with dichloromethane. The column was eluted with 2-5% methanol/CH₂Cl₂. The right fractions were pooled and evaporated to yield 2.98 g of Y3.

Synthesis of Y4: To a solution of 2.50 g of **Y3** in 40 ml of dichloromethane cooled in an ice bath was added 7.1 ml of trimethylsilane, followed by adding 17.5 ml of trifluoroacetic acid. The resulting reaction mixture was warmed to room temperature after 5 min of stirring at the same temperature. The reaction mixture was stirred at room temp for 7.5 hours. The solvent was removed. The residue was

dissolved in 5 ml of dichloromethane and loaded onto a column containing 25 g of silica gel packed with dichloromethane. The column was eluted with 0-2.5% methanol/CH₂Cl₂. The fractions were pooled and evaporated to yield 2.18 g of **Y4**.

Synthesis of Y5: 0.98 g of methyl N-(2-MMT-aminoethyl) glycinate was dissolved in 7 ml of dimethylformaide (DMF). To this solution was added 0.329 g of 3,4-dihydro-3-hydorxy-4-oxo-1,2,3-benzotriazine and 0.51 ml of 4-ethylmorpholine. A solution of 1.0 g of Y4 in 7 ml of DMF was added to the reaction mixture, followed by adding 0.38 ml of N,N'-diisopropylcarbodiimide. The reaction was stirred at room temperature for 20 hours. The solvent was then removed and the residue was dissolved in dichloromethane. The solution was washed with a saturated sodium chloride solution, and dried over sodium sulfate. The solvent was evaporated to about 5 ml for column chromatography. The crude mixture was loaded onto a 20 g silica gel column packed with 1% TEA/CH₂Cl₂. The column was eluted with 0-2% methanol/1%TEA/CH₂Cl₂. Evaporation of the solvent afforded 0.97 g of Y5.

Synthesis of Y6: 0.97 g of Y5 was dissolved in 10ml of dioxane and 2ml water. The pH of the mixture was adjusted to 11 with 1M NaOH. The reaction was stirred for two hours at 0°C. The hydrolysis reaction was monitored by TLC (CH₃OH:CH₂Cl₂). Upon the completeness of the hydrolysis, the pH of the mixture was adjusted to 5 with 2M potassium hydrogen sulfate. The mixture was extracted three times with CH₂Cl₂ (3 X 200 ml) and the combined extracts dried over sodium sulfate. The solution was evaporated to about 5 ml for column chromatography. Silica gel (20 gm) was packed with 1% triehtylamine in dichloromethane. The sample solution was loaded and the column was eluted with a 5-10% methanol./1% TEA/dichloromethane. The fractions containing the right product was pooled, evaporated and co-evaporated with pyridine and toluene in order to remove the triehthylamine to give 0.8 g of Y6.

25

30

35

20

5

10

15

Synthesis of Y7: To a solution of 0.8 g of Y6 in 80 ml of acetonitrile was added 0.61 g of 2-mitrobenzaldoxime and 0.37 g of 1,1,3,3-tetramethylguandine. The resulting solution was stirred at room temperature for 6 hours. The solvent was removed. The residue was dissolved in dichloromethane and washed with a saturated NaCl solution. Silica gel (20 g) was packed with 1% triehtylamine in dichloromethane. The crude residue was dissolved in 5 ml of dichloromethane and loaded onto the column. The column was eluted with 0-5% methanol/1%TEA/CH₂Cl₂. The fractions containing the product were pooled and concentrated to give 150 mg of product. The product was then dissolved in 100 ml of dichloromethane. The solution was washed with 10 ml of water and 10 ml of 10% tetrabutylammonium hydroxide. The organic layer was separated and dried over sodium sulfate and evaporated to give 200 mg of Y7.

-95-

CLAIMS

We claim:

- 1. A composition comprising:
 - a) a first electron transfer moiety comprising an electrode;
- 5 b) a first single stranded nucleic acid;
 - c) a second electron transfer moiety covalently attached to said first nucleic acid; and
 - d) a conductive oligomer covalently attached to both said electrode and said first nucleic acid.
 - 2. A composition comprising:
 - a) a first electron transfer moiety comprising an electrode;
 - b) a first single stranded nucleic acid;
 - c) a conductive oligomer covalently attached to both said electrode and said first nucleic acid;
 - d) a second electron transfer moiety covalently attached to a second single stranded nucleic acid.
 - 3. A composition according to claim 1 or 2 wherein said conductive oligomer has the formula:

$$\frac{-\left(-\left(\theta\right)_{g}^{2}\right)_{c}^{2}\left(-\left(\frac{1}{2}\right)_{g}^{2}\right)}{\left(-\left(\frac{1}{2}\right)_{g}^{2}\right)_{c}^{2}\left(-\left(\frac{1}{2}\right)_{g}^{2}\right)}$$

20 wherein

10

15

Y is an aromatic group;

n is an integer from 1 to 50;

g is either 1 or zero;

e is an integer from zero to 10; and

25 m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus.

A composition according to claim 1 or 2 wherein said conductive oligomer has the formula:

$$-\left(-c-c-c\right)^{m}$$

wherein

n is an integer from 1 to 50;

35 m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene.

- 5 A composition according to claim 1, 2, 3 or 4 wherein said electrode further comprises a monolayer of passivation agent.
 - 6. A composition according to claim 1, 2, 3, 4 or 5 wherein at least one of said nucleic acid is a nucleic acid analog.
 - 7. A composition according to claim 6 wherein said nucleic acid analog is a peptide nucleic acid.
 - 8. A composition according to claim 1, 2, 3, 4, or 5 further comprising a hybridization indicator.
- 9. A method of detecting a target sequence in a nucleic acid sample comprising:

 a) applying a first input signal to a hybridization complex comprising said target sequence,
 which if present, is hybridized to at least a first probe nucleic acid comorising a covalently
 attached conductive oligomer which is also covalently attached to a first electron transfer moiety comprising an electrode, wherein said hybridization complex has a covalently attached

 20 second electron transfer moiety; and
 - b) detecting electron transfer between said electrode and said second electron transfer moiety as an indication of the presence or absence of said target sequence.
 - 10. A method according to claim 9 wherein said conductive oligomer has the formula:

25

10

$$\frac{-\left(-\sqrt{\left(B\right)_{g}}D\right)_{e}}{\left(-\sqrt{2}\right)_{g}}$$

wherein

Y is an aromatic group;

n is an integer from 1 to 50;

30 g is either 1 or zero;

e is an integer from zero to 10; and

m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein

35 the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus.

11. A method according to claim 9 wherein said conductive oligomer has the formula:

wherein

10

20

25

30

5 n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene.

- 12. A method according to claim 9, 10 or 11 wherein said first input signal is selected from the group consisting of applied potential and photoactivation.
- 13. A method according to claim 9, 10, 11 or 12 wherein said first input signal comprises an AC component and a non-zero DC component.
 - 14. A method according to claim 9, 10, 11, 12 or 13 wherein said first input signal comprises an AC component at a first frequency and a non-zero DC component, and said method further comprises applying a second input signal comprising an AC component at at least a second frequency and a non-zero DC component.
 - 15. A method according to claim 9, 10, 11, 12, 13 or 14 wherein said first input signal comprises an AC component and a first non-zero DC component, and said method further comprises applying a second input signal comprising an AC component and a second non-zero DC component.
 - 16. A method according to claim 9, 10, 11, 12, 13, 14 or 15 wherein said first input signal comprises an AC component at a fist voltage amplitude and said method further comprises applying a second input signal comprising an AC component at a second voltage amplitude.
 - 17. A method according to claim 9, 10, 11, 12, 13, 14, 15 or 16 wherein said input signal includes the use of a co-redoxant.
- 18. A method according to claim 9, 10, 11, 12, 13, 14, 15, 16 or 17 wherein said input signal includes the use of a hybridization indicator.

19. A method according to claim 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 wherein detection of electron transfer occurs by receiving an output signal characteristic of electron transfer through said hybridization complex, and said output signal is selected from the group consisting of current, voltage, or phase shift between said input and said output signals.

5

- 20. A method of making a composition according to claim 1, 2, 3, 4, 5, 6 7 or 8 comprising attaching a conductive oligomer to a nucleic acid, and attaching said conductive oligomer to said electrode, in any order.
- 21. A composition comprising a conductive oligomer covalently attached to a nucleoside, wherein said conductive oligomer is selected from the group consisting of:

i)

$$-\left(-\left(\theta\right)_{g}\right)_{c}$$

wherein

15

Y is an aromatic group;

n is an integer from 1 to 50;

g is either 1 or zero;

e is an integer from zero to 10;and

m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus; or

ii)

25 wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

d is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not

alkane.

22. A composition comprising:

a) a solid support comprising a monolayer of passivation agent;

b) a nucleic acid comprising at least one nucleoside, wherein said nucleic acid is covalently attached to said solid support with a linker selected from the group selected from:

 $\frac{--\left(-\frac{1}{2}\left(8\right)_{g}}{--\left(8\right)_{g}} \left(-\frac{1}{2}\right)_{g}$

5 wherein

Y is an aromatic group;

n is an integer from 1 to 50;

g is either 1 or zero;

e is an integer from zero to 10; and

10 m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus; and

15

wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and
G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not alkane.

- 25 23. A composition according to claim 21 or 22 further comprising a hybridization indicator.
 - 24. A composition comprising:
 - a) an electrode;
 - b) at least one metallocene; and
- 30 c) a conductive oligomer covalently attached to both said electrode and said metallocene, wherein said conductive oligomer is selected from the group consisting of:

$$\frac{-\left(-c-c-c\right)_{n}\left(-1\right)_{m}}{\left(-c-c\right)_{n}\left(-1\right)_{m}}$$

wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene; and

5

$$\frac{1}{\left(-\frac{1}{2}\left(\theta\right)_{g}}\right)^{2}\left(-\frac{1}{2}\left(\theta\right)_{g}\right)^{2}\left(-\frac{1}{2}\left(\theta$$

wherein

Y is an aromatic group;

n is an integer from 1 to 50;

g is either 1 or zero;

e is an integer from zero to 10;and

m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety,

wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or

phosphorus.

25. A peptide nucleic acid with at least one chemical substituent covalently attached to the α -carbon of a subunit of said peptide nucleic acid.

20

15

26. A peptide nucleic acid with at least one chemical substituent covalently attached to an internal subunit of said peptide nucleic acid.

25

27. A peptide nucleic acid according to claim 26 said attachment is to a base of said subunit.

29. A composition according to claim 25, 26, 27 or 28 wherein said chemical substituent is a label.

28. A peptide nucleic acid according to claim 26 said attachment is to the backbone of said subunit.

SCH₂CH₂

(1) mCPBA/CH₂Ch₂AT
$$0$$
°C

(2) Ca(OH)₂

(3) (CF₃CO)₂ O / CH₂Cl₂ / Refluxing (5) 4-Vinylpyridine (2) Ca(OH)₂

(4) TEA/CH₃OH

Bezene / Refluxing (5) 4-Vinylpyridine (2) Ca(OH)₂ / Cal/ E₂NH

COMPOUND #1

COMPOUND #1

COMPOUND #3

COMPOUND #4

COMPOUND #5

FIG. 1A

FIG. 1A

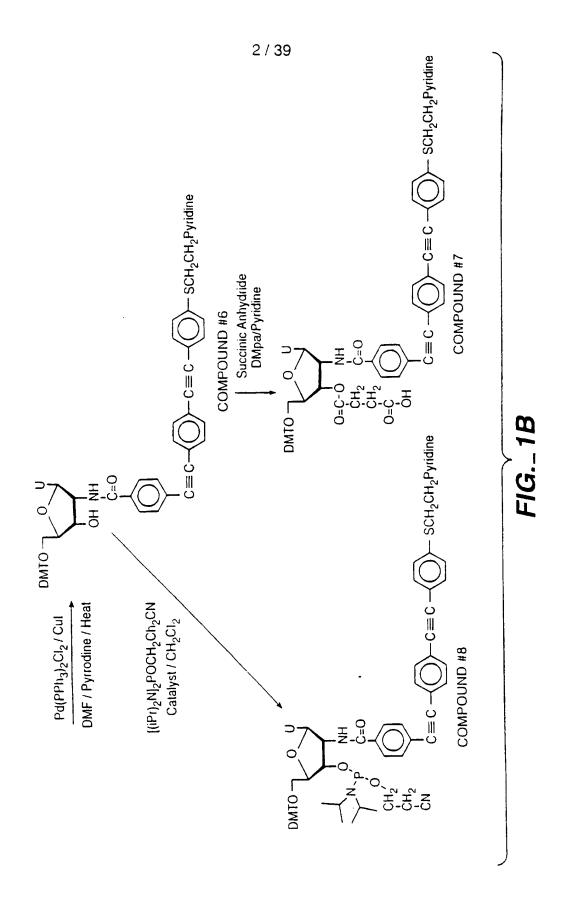
SCH₂CH₂CH₂ - Pyr

COMPOUND #5

C=C-TMS

C=

SUBSTITUTE SHEET (RULE 26)



SUBSTITUTE SHEET (RULE 26)

SUBSTITUTE SHEET (RULE 26)

$$H = \bigcirc = \bigcirc - SCH_2CH_2Pyridine / DMFO / DMF$$

$$AlC_3 / CH_3 COCI / CH_2 CI_2$$

$$AlC_3 / CH_3 COCI / CH_2 CI_2$$

$$COCH_3$$

$$LDA / TMS CI$$

$$LDA / TMS CI$$

$$COCH_3$$

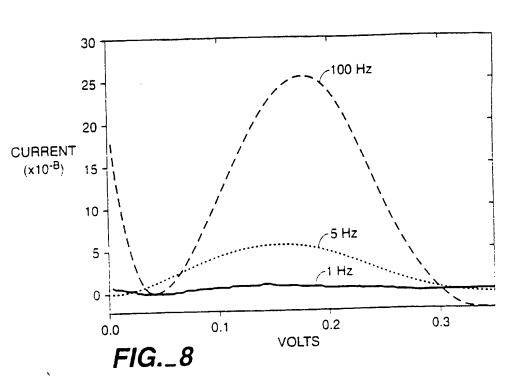
$$LDA / TMS CI$$

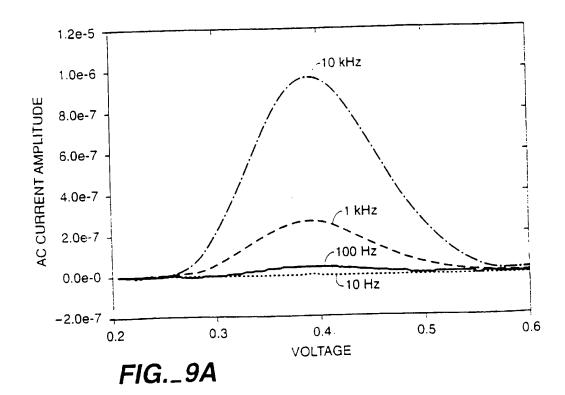
$$I MS$$

$$I - \bigcirc = - TMS$$

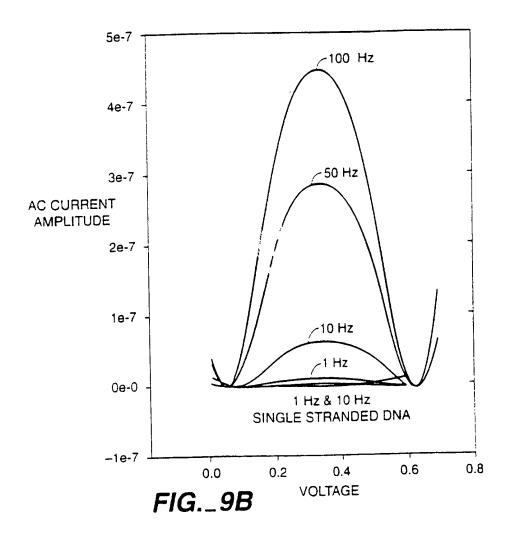
SUBSTITUTE SHEET (RULE 26)





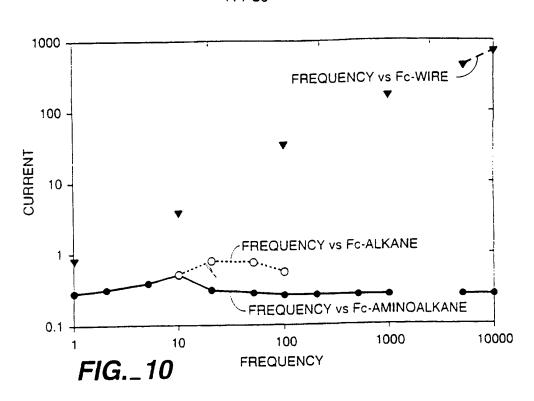


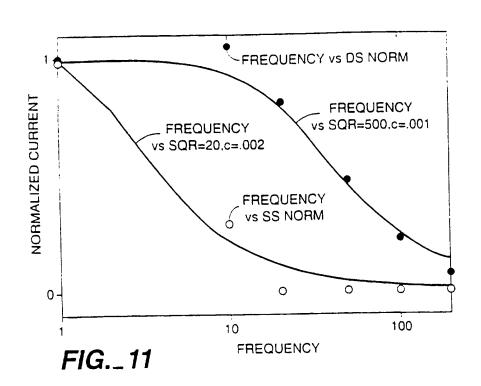
10/39



PCT/US97/20014 *

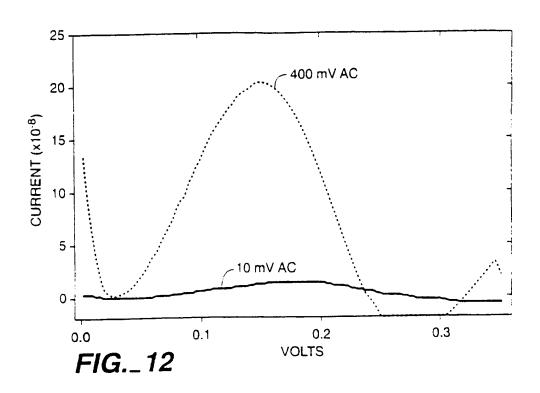
11 / 39

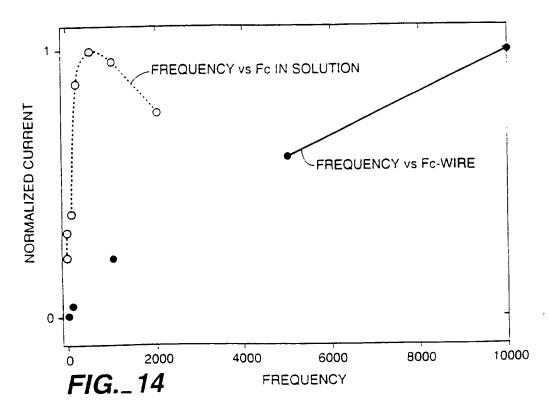


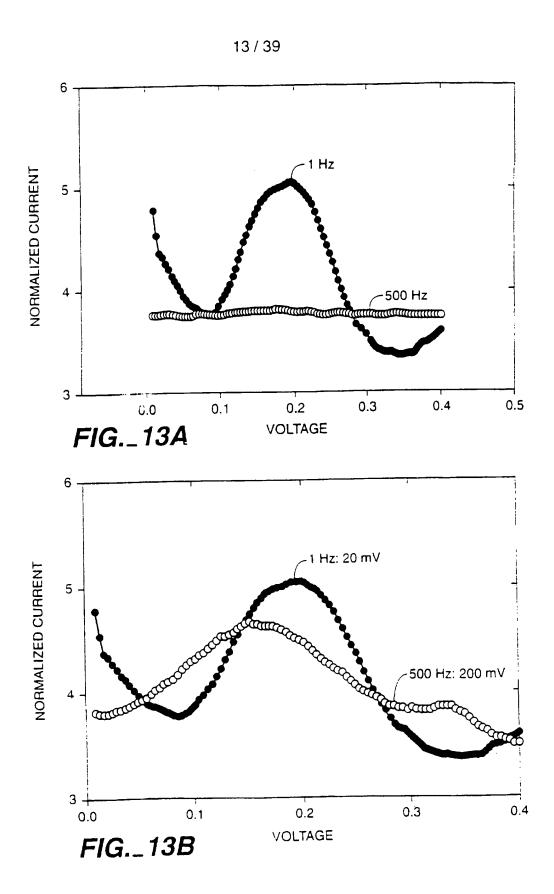


SUBSTITUTE SHEET (RULE 26)

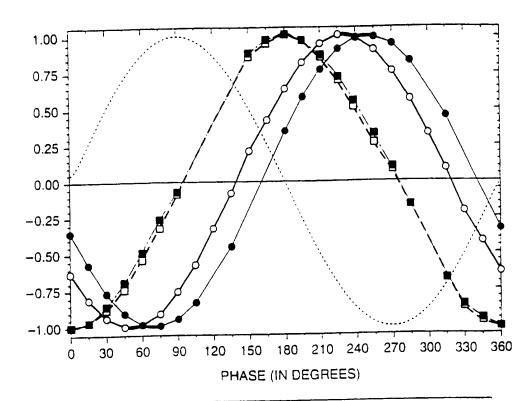








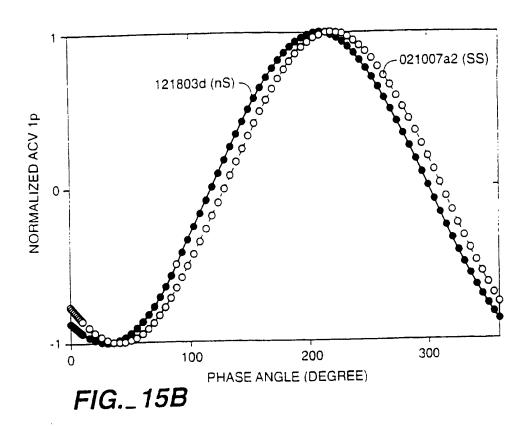
SUBSTITUTE SHEET (RULE 26)



- -O- Fc-ALKANE + HO-INSULATOR(#1) [0213_21] MAX @ 182 DEG
- Fc-ALKANE + HO-INSULATOR (#2) [0213_55] MAX @ 179 DEG.
- -D Fc-WIRE (DISULFIDE) + HO-INSULATOR. 2-STEP PROCESS [g010901]
- MAX @ 230 DEG.
- -■- Fc-WIRE (PROTECTED) + HO-INSULATOR. 3 STEP PROCESS [g010834]
- MAX @ 250 DEG.

..... DRIVING FORCE

FIG._15A



SUBSTITUTE SHEET (RULE 26)

18 / 39

19/39

FIG._17F

* TR.

T. Career

$$1 = \bigcirc = \bigcirc = \bigcirc -1$$

$$0.09 \text{ M NaCH} \quad H = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$0.09 \text{ M NaCH} \quad H = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$0.09 \text{ M NaCH} \quad H = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -1$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$1 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

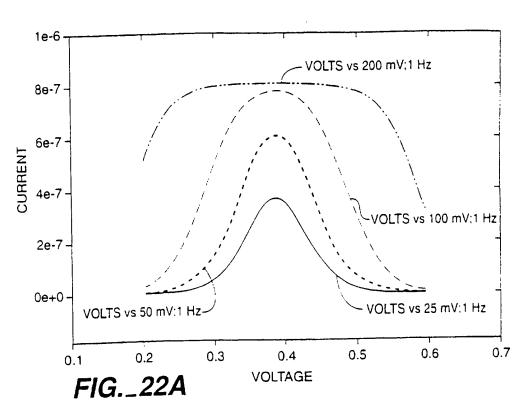
$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

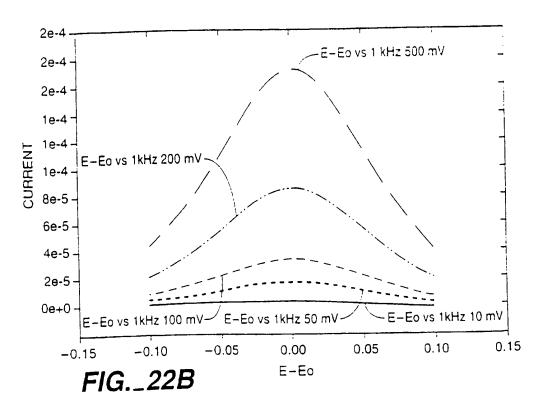
$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 - \bigcirc -2 - \bigcirc -2$$

$$2 = \bigcirc -2 -2$$







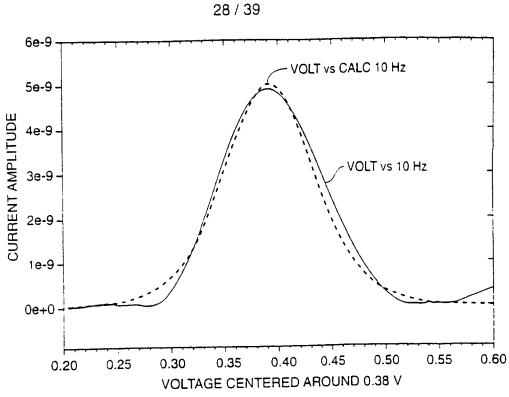
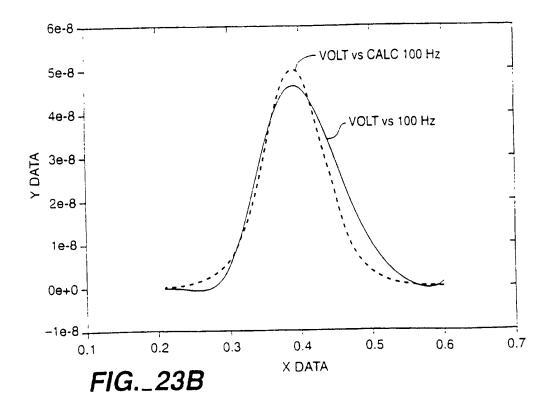


FIG._23A



29 / 39

$$\begin{array}{c} \text{NH}_2 \\ \text{N} \\ \text{N} \\ \text{NH} \end{array} + \begin{array}{c} \text{OCH}_3 \\ \text{Pyridine} \end{array} \\ \text{CH}_3 \\ \text{O} \\ \text{CI} \\ \text{CH}_3 \\ \text{O} \\ \text{CI} \\ \text{N} \\ \text{N}$$

FIG._24

FIG._25

FIG._26

FIG._27



SUBSTITUTE SHEET (RULE 26)

SUBSTITUTE SHEET (RULE 26)

SUBSTITUTE SHEET (RULE 26)

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM

GEBIET DES PATENTWESENS

PCT

REC'D 26 MAR 2001 WIPO PCT

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

(Artikel 36 und Regel 70 PCT)

	(Artikel 36 und R	egei 70 PC	' <i>)</i>				
Aktenzeichen des Anmelders oder Anwalts	WEITERES VORGEH	siehe Mitteil EN vorläufigen	ung über die Übersendung des internationalen Prüfungsberichts (Formblatt PCT/IPEA/416)				
FRI006/00							
Internationales Aktenzeichen	Internationales Anmeldedatu	ım(Tag/Monat/Jahr)	Prioritätsdatum (Tag/Monat/Tag)				
PCT/EP00/00084	07/01/2000		18/01/1999				
Internationale Patentklassifikation (IPK) oder C12Q1/68 Anmelder	nationale Klassifikation und IP	К					
FRIZ BIOCHEM GMBH							
Behörde erstellt und wird dem Ann	 Dieser internationale vorläufige Prüfungsbericht wurde von der mit der internationalen vorläufigen Prüfung beauftragten Behörde erstellt und wird dem Anmelder gemäß Artikel 36 übermittelt. 						
2. Dieser BERICHT umfaßt insgesan							
und/oder Zeichnungen, die ge Behörde vorgenommenen Bei	Außerdem liegen dem Bericht ANLAGEN bei; dabei handelt es sich um Blätter mit Beschreibungen, Ansprüchen und/oder Zeichnungen, die geändert wurden und diesem Bericht zugrunde liegen, und/oder Blätter mit vor dieser Behörde vorgenommenen Berichtigungen (siehe Regel 70.16 und Abschnitt 607 der Verwaltungsrichtlinien zum PCT). Diese Anlagen umfassen insgesamt 10 Blätter.						
3. Dieser Bericht enthält Angaben zu folgenden Punkten: Grundlage des Berichts Priorität							
III	s Gutachtens über Neuheit	, erfinderische Täi	igkeit und gewerbliche Anwendbarkeit				
IV	nkeit der Erfindung						
V ⊠ Begründete Feststellu gewerblichen Anwend	ung nach Artikel 35(2) hinsi dbarkeit; Unterlagen und Ei	chtlich der Neuher rklärungen zur Stü	t, der erfinderischen Tätigkeit und der tzung dieser Feststellung				
VI 🛛 Bestimmte angeführte	e Unterlagen		,				
VII Bestimmte Mängel de	VII Bestimmte Mängel der internationalen Anmeldung						
VIII Bestimmte Bemerkungen zur internationalen Anmeldung							
Datum der Einreichung des Antrags		Datum der Fertigste	llung dieses Berichts				
09/07/2000		21.03.2001					
Name und Postanschrift der mit der interna Prüfung beauftragten Behörde: Europäisches Patentamt D-80298 München	3	Bevollmächtigter Be Favre, N	diensteter				
Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465 Tel. Nr. +49 89 2399 7363							

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/00084

I.	Grundlage des Berichts
	Dieser Bericht wurde erstellt auf der Grundlage (Ersatzblätter, die dem Anmeldeamt auf eine Al Artikel 14 hin vorgelegt wurden, gelten im Rahmen dieses Berichts als "ursprünglich eingereich Artikel 14 hin vorgelegt wurden, gelten im Rahmen dieses Berichts als "ursprünglich eingereich

•	iruno	alage des bellici			Anmoldoamt auf A	ine Aufforderuna nach	
	Artike nicht l	1 4 4 hin voraelea	erstellt auf der Grundlage (<i>Ersat</i> It wurden, gelten im Rahmen die Ie keine Änderungen enthalten.): n:	ses benons a	m Anmeldeam au e Als "ursprünglich einge	ereicht" und sind ihm	
	1-61		ursprüngliche Fassung				
	Pater	ntansprüche, Nr	::				
	1-71		eingegangen am	14/11/2000	mit Schreiben vom	07/11/2000	
	Zeict	nnungen, Blätte	r:				
	1/6-6	/6	ursprüngliche Fassung				
			der Beschreibung, Seiten:				
	1, eir	ngereicht mit Sch	reiben vom 07.07.2000.				
2.	Hinsichtlich der Sprache: Alle vorstehend genannten Bestandteile standen der Behörde in der Sprache, in der die internationale Anmeldung eingereicht worden ist, zur Verfügung oder wurden in dieser eingereicht, sofern unter diesem Punkt nichts anderes angegeben ist.						
Die Bestandteile standen der Behörde in der Sprache: zur Verfügung bzw. wurden in dieser Sprache eingereicht; dabei handelt es sich um							
		Regel 23 1(b)).	Übersetzung, die für die Zweck			gereicht worden ist (nach	
		die Veröffentlichungssprache der internationalen Anmeldung (nach Regel 48.3(b)). die Sprache der Übersetzung, die für die Zwecke der internationalen vorläufigen Prüfung eingereicht worder					
		ist (nach Regel	55.2 und/oder 55.3).				
3	Hins inte	rnationale vorläu	er internationalen Anmeldung off fige Prüfung auf der Grundlage	des dequerizp	, otonomo a assas g	inosäuresequenz ist die t worden, das:	
		in der internatio	nalen Anmeldung in schriftlicher	Form enthalte	en ist.	a de la companya de l	
		zusammen mit	der internationalen Anmeldung i	n computerles	barer Form eingereich	nt worden ist.	
	\boxtimes	bei der Behörde	e nachträglich in schriftlicher For	m eingereicht	worden ist.		
	×	bei der Behörde	e nachträglich in computerlesba	rer Form einge	ereicht worden ist.	at über den	
	Ø	Offenharungen	daß das nachträglich eingereich ehalt der internationalen Anmeld	Jung iin Aninei	dezemparikt illi aaaaga	,	
	×	Die Erklärung	daß die in computerlesbarer For coll entsprechen, wurde vorgele	rm erfassten Ir	nformationen dem sch	riftlichen	

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/00084

4.	Aufgrund der Änderungen sind folgende Unterlagen fortgefallen:								
		Beschreibung,	Seiten:						
		Ansprüche,	Nr.:						
		Zeichnungen,	Blatt:						
5.	 Dieser Bericht ist ohne Berücksichtigung (von einigen) der Änderungen erstellt worden, da diese aus den angegebenen Gründen nach Auffassung der Behörde über den Offenbarungsgehalt in der ursprünglich eingereichten Fassung hinausgehen (Regel 70.2(c)). 						den ich		
		(Auf Ersatzblätter, d beizufügen).	ie solche Änderu	ngen enthalte	n, ist unte	r Punkt 1 hinz	:uweisen;sie :	sind diesem B	3ericht
6.	Etw	aige zusätzliche Berr	nerkungen:						
V.	Beg gev	gründete Feststellur verblichen Anwendb	ng nach Artikel 3 parkeit; Unterlag	35(2) hinsicht Jen und Erklä	lich der N rungen z	leuheit, der e ur Stützung (erfinderische dieser Festst	en Tätigkeit u tellung	nd de
1.	Fes	ststellung							
	Nei	uheit (N)	Ja: Neir	Ansprüchen: Ansprüche	1-71				
	Erfi	nderische Tätigkeit (E	ET) Ja: Neir	Ansprüchen: Ansprüche	1-71				
	Ge	werbliche Anwendbai	rkeit (GA) Ja: Neir	Ansprüchen: Ansprüche	1-71				
2.		terlagen und Erklärur he Beiblatt	ngen					·	
VI	. Be	stimmte angeführte	Unterlagen						
1.	Be	stimmte veröffentlicht	e Unterlagen (Re	egel 70.10)	•	•			

siehe Beiblatt

und / oder

2. Nicht-schriftliche Offenbarungen (Regel 70.9)

Zu Punkt I Grundlage des Berichts

Das Sequenzprotokol, welches mit dem Schreiben vom 07.07.2000 eingereicht wurde, ist nicht Bestandteil der Anmeldung (Regel 13*ter.*1 f) PCT).

Zu Punkt V

Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststeller.g

Dokument D1 (US-A-5 770 369) offenbart Nukleinsäure-Oligomere, die durch kovalente Anbindung von redoxaktiven Verbindungen, wie zum Beispiel Übergangsmetallkomplexe, modifiziert sind (Abstract). Die Übergangsmetallkomplexe können unter anderem die Metalle Cd, Mg, Cu, Co, Pd, Zn, Fe, Ru, Rh, Os, Re, Pt, Sc, Ti, V, Cr, Mn, Ni, Mo, Tc, W und Ir enthalten (Spalte 7, Zeile 35 bis 48). Als Liganden der Metalle werden in D1 unter anderem Pyridin, Pyrazin, Bipyridin, Phenanthrolin, 9,10-Phenanthrenchinondiimin, Porphyrine und substituierte Derivate der Porphyrine (Spalte 7, Zeile 49 bis 62) genannt. Alternativ zu den Übergangsmetallkomplexen können auch andere organische Elektronendonoren bzw. -akzeptoren kovalent an die Nukleinsäuren gebunden werden (Spalte 8, Zeile 25 bis 27), zum Beispiel Riboflavin, Chinone, Porphyrine, ..., (Spalte 8, Zeile 27 bis 43).

Gemäß einer Ausführungsform von D1 werden die Nukleinsäuren an eine Elektrode gebunden (Spalte 8, Zeite 47 bis 50), wodurch ein Elektronentransport zwischen der Elektrode und der Nukleinsäure direkt über eine kovalente Bindung erfolgen kann (Spalte 9, Zeile 56 bis 58).

Somit stellt D1 ein System zur Verfügung, bei dem ein mit einem redoxaktiven organischen Molekül modifizertes Oligonukleotid an eine Elektrode gebunden ist (Spalte 11, Zeile 1 bis 12). Das Vorliegen einer doppelsträngigen DNA kann durch Vergleich der Elektronentransferraten von unhybridisierter Probe und

hybridisierter Probe erfolgen (Spalte 12, Zeile 44 bis 48). Zur Detektion des Elektronentransfers wird unter anderem die Amperometrie vorgeschlagen, was einen Vergleich der Stromstärke bei Vorliegen von hybridisierter bzw. unhybridisierter DNA ermöglicht (Spalte 27, Zeile 20 bis 47). Zur Initiierung der Elektronenübertragung kann Bestrahlung mit Licht angewendet werden (Spalte 23, Zeile 50 bis 53).

- 1.1 Dokument D2 (WO-A-98 20162) offenbart Nukleinsäuren, die durch kovalente Anbindung von redoxaktiven Verbindungen, wie zum Beispiel Übergangsmetall-komplexe, modifiziert sind (Seite 37, Zeile 22 und Seite 25, Zeile 27 bis 33). Als Liganden der Metalle werden unter anderem Pyridin, Pyrazin, Bipyridin, Phenanthrolin, ..., genannt (Seite 26, Zeile 14 bis 29). Alternativ zu den Übergangsmetallkomplexen können andere organische Elektronen-donoren bzw. -akzeptoren kovalent an die Nukleinsäuren gebunden werden (Seite 37, Zeile 29 bis 31), zum Beispiel Riboflavin, Chinone, Porphyrine, ... (Seite 37, Zeile 30 bis Seite 38, Zeile 5).
 - Die modifizierten Nukleinsäuren werden über ein leitfähiges Polymer an eine Elektrode gebunden (Anspruch 1), wodurch ein Elektronentransport zwischen der Elektrode und der Nukleinsäure erfolgen kann (Anspruch 9).
 - Somit stellt D2 ein System zur Verfügung, bei dem ein mit einem redoxaktiven organischen Molekül modifiziertes Oligonukleotid über ein leitfähiges Polymer an eine Elektrode gebunden ist (Anspruch 1 + 9). Das Vorliegen einer doppelsträngigen DNA kann durch erhöhten Elektronentransfer von der DNA auf die Elektrode detektiert werden (Seite 52, Zeile 21 bis 24). Zur Detektion des Elektronentransfers wird unter anderem die Amperometrie vorgeschlagen (Seite 56, Zeile 18 bis 25).
 - 1.2 Dokument D3 (DE-A-42 16 696) offenbart ein Verfahren zur Durchführung von Assays, die unter anderem auf Wechselwirkungen zwischen DNA-Strängen beruhen. Die Detektion basiert zum Beispiel auf einer Messung der Stromstärke, wobei stabile redox-markierte Analytmoleküle Verwendung finden (Abstract). Dazu wird ein DNA-Strang mit einem stabilen Redox-System kovalent verbunden (Spalte 4, Zeile 6 bis 14). Als Redox-Systeme werden Ferrocenderivate, Ruthenium-komplexe, Hydrochinone, Hexacyanoferrat (II/III), Jod/Jodid oder ähnliche vorgeschlagen (z.B. Anspruch 22).

- 1.3 Weder eines der Dokumente D1 bis D3, noch eines der vorhandenen Dokumenten des Standes der Technik, offenbart oder legt ein durch eine redoxaktive Einheit modifiziertes Nukleinsäure-Oligomer nahe, bei dem die redoxaktive Einheit, wie von unabhängigen Anspruch 1 der vorliegenden Anmeldung gefordert, wenigstens ein Elektron-Donor-Molekül und wenigstens ein Elektron-Akzeptor-Molekül enthält, wobei Elektron-Donor-Molekül und Elektron-Akzeptor-Molekül nicht durch ein Nukleinsäure-Oligomer miteinander verbunden sind (siehe auch Seite 10, Zeile 18 bis 19, der Beschreibung).
- 1.4 Die Vorteile einer solches modifiziertes Nukleinsäure-Oligomers gegenüber den im Stand der Technik beschriebenen modifizierten Nukleinsäure-Oligomeren sind, unter anderen, dass die Übertragung einer Ladung aus dem angeregten Zustand nicht direkt auf die DNA, sondern direkt auf ein weiteres Molekül, das innerhalb der redoxaktiven Einheit vorhanden ist, geschieht. Erst von dem Akzeptor erfolgt die Übertragung der Ladung auf die DNA. Der Vorteil dieser aufeinanderfolgenden Übertragungen liegt in der Lebensdauer des Zustands der redoxaktiven Einheit nach der ersten Übertragung der Ladung. Diese ist wenigstens 10³x, im Fall des photosynthetischen bakteriellen Reaktionszentrums (Seite 32) sogar 10³x länger als die des ursprünglichen angeregten Zustandes des Donors. Um denselben Faktor erhöht sich auch die Wahrscheinlichkeit der Übertragung der Ladung auf die DNA, was drastische Auswirkungen auf die intensität des durch die DNA geleiteten Stroms hat.
- 1.5 Anspruch 1 ist daher neu und erfinderisch im Sinne von Artikeln 33(2) und 33(3) PCT.
- 1.6 Die abhängigen Ansprüche 2-27 definieren weitere spezifische Ausführungsformen des Anspruchs 1 und sind daher auch neu und erfinderisch im Sinne von Artikeln 33(2) und 33(3) PCT.
- Patentansprüche 28-36 definieren Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers wie in Ansprüchen 1-27 beschrieben.
 Da diese modifizierten Nukleinsäure-Oligomers neu und erfinderisch sind, sind auch die in Ansprüchen 28-36 beschrieben Verfahren neu und erfinderisch im

Sinne von Artikeln 33(2) und 33(3) PCT.

- Dasselbe gilt auch für die in Patentansprüchen 37-57 definierten modifizierten leitfähigen Oberflächen, die dadurch gekennzeichnet sind, daß wenigstens ein modifiziertes Nukleinsäure-Oligomer wie in Ansprüchen 1-27 definiert, an eine solche leitfähige Oberfläche gebunden ist.
 - Patentansprüche 37-57 erfüllen daher die Erfordernisse des PCT in bezug auf Neuheit und erfinderische Tätigkeit im Sinne von Artikeln 33(2) und 33(3) PCT.

٠.

 Analog dazu, sind die in Patentansprüchen 58-71 definierte Verfahren zur Herstellung solcher modifizierten leitfähigen Oberflächen ebenfalls neu und erfinderisch im Sinne von Artikeln 33(2) und 33(3) PCT.

Zu Punkt VI Bestimmte angeführte Unterlagen

Bestimmte veröffentlichte Unterlagen (Regel 70.10)

Anmelde Nr. Patent Nr.	Veröffentlichungsdatum (Tag/Monat/Jahr)	Anmeldedatum (Tag/Monat/Jahr)	Prioritätsdatum (zu Recht beansprucht) (Tag/Monat/Jahr)
WO-A-99 51778	14.10.1999	07.04.1999	08.04.1998
WO-A-00 31101	02.06.2000	19.11.1999	23.11.1998 ⁻ / 29.04.1999 ⁻

Bei Eintritt in die Regionalphase könnten obengenannte Dokumente für die Beurteilung der Neuheit relevant werden.

Die Gültigkeit des Prioritätsanspruch wurde nicht überprüft

Neue Patentansprüche 1 bis 71

- Durch kovalente Anbindung einer redoxaktiven Einheit modifiziertes Nukleinsäure-Oligomer, dadurch gekennzeichnet, dass die redoxaktive Einheit wenigstens ein Elektron-Donor-Molekül und wenigstens ein Elektron-Akzeptor-Molekül enthält, wobei Elektron-Donor-Molekül und Elektron-Akzeptor-Molekül nicht durch Nukleinsäure-Oligomere miteinander verbunden sind.
- 1, dadurch Anspruch Nukleinsäure-Oligomer nach Modifiziertes 2. gekennzeichnet, dass die redoxaktive Einheit wenigstens einen redoxaktiven, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptorverknüpften, Elektron-Donor-Molekül wobei wenigstens ein Komplex enthält, redoxaktiven Einheit und wenigstens ein Elektron-Akzeptor-Molekül der redoxaktiven Einheit durch eine oder mehrere Bindungen miteinander verbunden sind.
- Modifiziertes Nukleinsäure-Oligomer nach Anspruch 2, dadurch gekennzeichnet, dass es sich bei den Bindungen um kovalente Bindungen handelt.
- Anspruch 1. dadurch Nukleinsäure-Oligomer nach Modifiziertes 4. gekennzeichnet, dass die redoxaktive Einheit wenigstens einen redoxaktiven, Elekton-Donor-/Elektron-Akzeptorverknüpften, wenigstens bimolekularen Elektron-Donor-Molekül der ein wobei wenigstens Komplex enthält, redoxaktiven Einheit und wenigstens ein Elektron-Akzeptor-Molekül der redoxaktiven Einheit durch einen oder mehrere verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent verbunden sind.
- Modifiziertes Nukleinsäure-Oligomer nach Anspruch 4, dadurch gekennzeichnet, dass die verzweigten oder unverzweigten Molekülteile eine Kettenlänge von 1 - 20 Atomen aufweisen.
- Modifiziertes Nukleinsäure-Oligomer nach Anspruch 5, dadurch gekennzeichnet, dass die verzweigten oder unverzweigten Molekülteile eine Kettenlänge von 1 - 14 Atomen, aufweisen.

- 7. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit zusätzlich ein oder mehrere Makromoleküle umfaßt.
- 8. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei die redoxaktive Einheit das native oder modifizierte Reaktionszentrum von Photosynthese betreibenden Organismen ist.
- 9. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 8, wobei die redoxaktive Einheit das native oder modifizierte Reaktionszentrum von Photosynthese betreibenden Bakterien ist.
- Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass wenigstens eines der Elektron-Donor-Moleküle und Elektron-Akzeptor-Moleküle ein Farbstoff ist.
- 11. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 10, dadurch gekennzeichnet, dass es sich bei dem Farbstoff um ein Flavin, ein (Metallo-)Porphyrin, ein (Metallo-)Chlorophyll, ein (Metallo-)Bakteriochlorophyll oder ein Derivat dieser Farbstoffe handelt.
- Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass wenigstens eines der Elektron-Donor-Moleküle und Elektron-Akzeptor-Moleküle ein Nikotinsäureamid oder ein Chinon ist.
- 13. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 12, dadurch gekennzeichnet, dass es sich bei dem Chinon um ein Pyrrolo-Chinolin-Chinon (PQQ), ein 1,2-Benzochinon, ein 1,4-Benzochinon, ein 1,2-Naphtochinon, ein 1,4-Naphtochinon, ein 9,10-Anthrachinon oder eines derer Derivate handelt.
- 14. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass wenigstens eines der Elektron-Donor-Moleküle und Elektron-Akzeptor-Moleküle ein Charge-Transfer-Komplex ist.
- 15. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 14, dadurch gekennzeichnet, dass der Charge-Transfer-Komplex ein Übergangsmetall-Komplex ist.

- 16. Modifiziertes Nukleinsäure-Oligomer nach Anspruch gekennzeichnet, dass der Charge-Transfer-Komplex ein Ru(II)-, ein Fe(II)-, ein Os(II)- oder ein Co(II)-Komplex ist.
- 17. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergeher.

 Ansprüche, dadurch gekennzeichnet, dass das modifizierte Nukleinsäure.

 Oligomer sequenzspezifisch Einzelstrang-DNA, RNA und/oder PNA binden kann.
- 18. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 17, dadurch gekennzeichnet, dass das modifizierte Nukleinsäure-Oligomer ein Desoxyribonukleinsäure-, ein Ribonukleinsäure- oder ein Peptidnukleinsäure-Oligomer ist.
- 19. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit kovalent an eine der Phosphorsäure-Gruppen, an eine der Carbonsäure-Gruppen, an eine der Amin-Gruppen oder an einen Zucker des Nukleinsäure-Oligomer-Rückgrats gebunden ist.
- 20. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit kovalent an an eine Zucker-Hydroxy-Gruppe des Nukleinsäure-Oligomer-Rückgrats gebunden ist.
- 21. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 18, dadurch gekennzeichnet, dass die redoxaktive Einheit kovalent an eine Thiol-Gruppe, eine Hydroxy-Gruppe, eine Carbonsäure-Gruppe oder eine Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.
- 22. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 21, dadurch gekennzeichnet, dass die reaktive Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe der Base kovalent über einen verzweigten oder unverzweigten Molekülteil beliebiger Zusammensetzung und Kettenlänge an die Base gebunden ist, wobei die kürzeste durchgehende Verbindung zwischen der Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe und der Base ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen ist.

- 23. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 22, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen der Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe und der Base ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 - 14 Atomen ist.
- 24. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 19 bis 22, dadurch gekennzeichnet, dass die redoxaktive Einheit an ein Ende des Nukleinsäure-Oligomer-Rückgrats oder an eine endständige, modifizierte Base angebunden ist.
- 25. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit eine photoinduzierbar redoxaktive Einheit ist.
- 26. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 24, dadurch gekennzeichnet, dass die redoxaktive Einheit eine chemisch induzierbar redoxaktive Einheit ist.
- 27. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass mehrere redoxaktive Einheiten an das Nukleinsäure-Oligomer angebunden sind.
- 28. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers wie in einem der vorhergehenden Ansprüche definiert, dadurch gekennzeichnet, dass eine redoxaktive Einheit kovalent an ein Nukleinsäure-Oligomer angebunden wird.
- 29. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 28, dadurch gekennzeichnet, dass die redoxaktive Einheit durch kovalente Anbindung von wenigstens einem Elektron-Donor-Molekül an ein Nukleinsäure-Oligomer angebunden wird.
- 30. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 28, dadurch gekennzeichnet, dass die redoxaktive Einheit durch kovalente Anbindung von wenigstens einem Elektron-Akzeptor-Molekül an ein Nukleinsäure-Oligomer angebunden wird.

- 31. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 28, dadurch gekennzeichnet, dass die redoxaktive Einheit durch kovalente Anbindung wenigstens eines Makromoleküls oder durch kovalente Anbindung wenigstens eines Proteins an ein Nukleinsäure-Oligomer angebunden wird.
- 32. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach den Ansprüchen 29 bis 31, dadurch gekennzeichnet, dass die redoxaktive Einheit durch Zugabe von wenigstens einem Bestandteil ausgewählt aus der Gruppe bestehend aus Elektron-Akzeptor-Moleküle, Elektron-Donor-Moleküle, Makromoleküle und Proteine vervollständigt wird.
- 33. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 28 bis 32, dadurch gekennzeichnet, dass das Nukleinsäure-Oligomer durch eine oder mehrere Amidbildungen mit Amin- oder mit Säure-Gruppen der redoxaktiven Einheit, durch eine oder mehrere Esterbildungen mit Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit, durch Thioesterbildung mit Thio-Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit oder durch Kondensation einer oder mehrerer Amin-Gruppen des Nukleinsäure-Oligomers mit Aldehyd-Gruppen der redoxaktiven Einheit und anschließender Reduktion der entstandenen Kohlenstoff-Stickstoff-Doppelbindung an die redoxaktive Einheit gebunden wird.
- 34. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 28 bis 33, dadurch gekennzeichnet, dass wenigstens ein verzweigter oder unverzweigter Molekülteil beliebiger Zusammensetzung und Kettenlänge kovalent an die redoxaktive Einheit angebunden ist und die verzweigten oder unverzweigten Molekülteile eine reaktive Amin-, Hydroxy-, Thiol-, Säure- oder Aldehyd-Gruppe zur kovalenten Anbindung an ein Nukleinsäure-Oligomer aufweisen.
- 35. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 34, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen dem Nukleinsäure-Oligomer und der redoxaktiven Einheit ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen ist.
- 36. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 35, dadurch gekennzeichnet, dass die kürzeste durchgehende

Verbindung zwischen dem Nukleinsäure-Oligomer und der redoxaktiven Einheit ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 - 14 Atomen ist.

- 37. Modifizierte leitfähige Oberfläche, dadurch gekennzeichnet, dass wenigstens eine Art von modifiziertem Nukleinsäure-Oligomer gemäß einem der Ansprüche 1 bis 27 an eine leitfähige Oberfläche angebunden ist.
- 38. Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einem Metall oder einer Metallegierung besteht.
- 39. Modifizierte leitfähige Oberfläche nach Anspruch 38, dadurch gekennzeichnet, dass die Oberfläche aus einem Metall ausgewählt aus der Gruppe Platin, Palladium, Gold, Cadmium, Quecksilber, Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium und Mangan besteht.
- 40. Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einem Halbleiter besteht.
- 41. Modifizierte leitfähige Oberfläche nach Anspruch 38, dadurch gekennzeichnet, dass die Oberfläche aus einem Halbleiter ausgewählt aus der Gruppe Kohlenstoff, Silizium, Germanium und Zinn besteht.
- 42. Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einer binären Verbindung der Elemente der Gruppen 14 und 16, einer binären Verbindung der Elemente der Gruppen 13 und 15, einer binären Verbindung der Elemente der Gruppen 15 und 16, oder einer binären Verbindung der Elemente der Gruppen 11 und 17 besteht.
- 43. Modifizierte leitfähige Oberfläche nach Anspruch 42, dadurch gekennzeichnet, dass die Oberfläche aus einem Cu(I)-Halogenid oder einem Ag(I)-Halogenid besteht.
- 44. Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einer ternären Verbindung der Elemente der Gruppen 11, 13 und 16 oder einer ternären Verbindung Elemente der Gruppen 12, 13 und 16 besteht.

- 45. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 44, dadurch gekennzeichnet, dass die Anbindung der modifizierten Nukleinsäure-Oligomere an die leitfähige Oberfläche kovalent oder durch Chemi- oder Physisorption erfolgt.
- 46. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 45, dadurch gekennzeichnet, dass eine der Phosphorsäure-, Carbonsäure-, Amin- oder eine Zucker-Gruppe des Nukleinsäure-Oligomer-Rückgrats kovalent oder durch Chemi- oder Physisorption an die leitfähige Oberfläche angebunden ist.
- 47. Modifizierte leitfähige Oberfläche nach Anspruch 46, dadurch gekennzeichnet, dass eine Zucker-Hydroxy-Gruppe des Nukleinsäure-Oligomer-Rückgrats kovalent oder durch Chemi- oder Physisorption an die leitfähige Oberfläche angebunden ist.
- 48. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 45, dadurch gekennzeichnet, dass eine Thiol-Gruppe, eine Hydroxy-Gruppe, eine Carbonsäure-Gruppe oder eine Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers kovalent oder durch Chemi- oder Physisorption an die leitfähige Oberfläche angebunden ist.
- 49. Modifizierte leitfähige Oberfläche nach den Ansprüchen 46 bis 48, dadurch gekennzeichnet, dass das modifizierte Nukleinsäure-Oligomer über eine Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats oder über eine Gruppe einer endständigen, modifizierten Base an die leitfähige Oberfläche gebunden ist.
- 50. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 49, dadurch gekennzeichnet, dass an die leitfähige Oberfläche verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent oder durch Chemi- oder Physisorption angebunden sind und die modifizierten Nukleinsäure-Oligomere kovalent an diese Molekülteile angebunden sind.
- 51. Modifizierte leitfähige Oberfläche nach Anspruch 50, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen der leitfähigen Oberfläche und dem Nukleinsäure-Oligomer ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen ist.

- 52. Modifizierte leitfähige Oberfläche nach Anspruch 51, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen der leitfähigen Oberfläche und dem Nukleinsäure-Oligomer ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 12 Atomen, ist.
- 53. Modifizierte leitfähige Oberfläche nach den Ansprüchen 50 bis 52, dadurch gekennzeichnet, dass der verzweigte oder unverzweigte Molekülteil an eine Phosphorsäure-Gruppe, eine Carbonsäure-Gruppe, eine Amin-Gruppe oder eine Zucker-Gruppe des Nukleinsäure-Oligomer-Rückgrats oder eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.
- 54. Modifizierte leitfähige Oberfläche nach Anspruch 53, dadurch gekennzeichnet, dass der verzweigte oder unverzweigte Molekülteil an eine Zucker-Hydroxy-Gruppe des Nukleinsäure-Oligomer-Rückgrats angebunden ist.
- 55. Modifizierte leitfähige Oberfläche nach Anspruch 53 oder 54, dadurch gekennzeichnet, dass der verzweigte oder unverzweigte Molekülteil an eine Phosphorsäure-, Zucker-Hydroxy-, Carbonsäure- oder Amin-Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats oder an eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer endständigen, modifizierten Base gebunden ist.
- 56. Modifizierte leitfähige Oberfläche nach einem der Ansprüche 37 bis 55, dadurch gekennzeichnet, dass jeweils überwiegend eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.
- 57. Modifizierte leitfähige Oberfläche nach Anspruch 56, dadurch gekennzeichnet, dass jeweils ausschließlich eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.
- 58. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 37 bis 57 definiert, dadurch gekennzeichnet, dass wenigstens eine Art von modifizierten Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht wird.

- 59. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 37 bis 57 definiert, dadurch gekennzeichnet, dass wenigstens eine Art von Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht wird und anschließend eine Modifikation der Nukleinsäure-Oligomere durch ein Verfahren gemäß den Ansprüchen 28 bis 36 durchgeführt wird.
- 60. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach Anspruch 58 oder 59, dadurch gekennzeichnet, dass die Nukleinsäure-Oligomere oder die modifizierten Nukleinsäure-Oligomere mit dem dazu jeweils komplementären Nukleinsäure-Oligomerstrang hybridisiert werden und in Form des Doppelstranghybrids auf die leitfähige Oberfläche aufgebracht werden.
- 61. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 58 oder 59, dadurch gekennzeichnet, dass das Nukleinsäure-Oligomer oder das modifizierte Nukleinsäure-Oligomer in Gegenwart von weiteren chemischen Verbindungen, die ebenfalls an die leitfähige Oberfläche angebunden werden, auf die leitfähige Oberfläche aufgebracht wird.
- 62. Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybridisierungsereignissen, dadurch gekennzeichnet, dass wenigstens eine modifizierte leitfähige Oberfläche, wie in den Ansprüchen 37 bis 57 definiert, mit Nukleinsäure-Oligomeren in Kontakt gebracht wird und anschließend eine Detektion der elektrischen Kommunikation zwischen der redoxaktiven Einheit und der leitfähigen Oberfläche erfolgt.
- 63. Verfahren nach Anspruch 62, dadurch gekennzeichnet, dass die Detektion cyclovoltametrisch, amperometrisch oder durch Leitfähigkeitsmessung erfolgt.
- 64. Verfahren nach den Ansprüchen 62 oder 63, dadurch gekennzeichnet, dass die elektrochemische Detektion durch photoinduzierte Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktiven Einheit gestartet wird.
- 65. Verfahren nach Anspruch 64, dadurch gekennzeichnet, dass die Lichteinstrahlung zur photoinduzierten Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktiven Einheit auf einen Bereich der leitfähigen Oberfläche mit wenigstens einer Art von modifizierten Nukleinsäure-Oligomeren begrenzt wird.

- 66. Verfahren nach den Ansprüchen 64 oder 65, dadurch gekennzeichnet, dass das nach Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entstandene oxidierte Elektron-Donor-Molekül der photoinduzierbar redoxaktiven Einheit durch eine geeignete, freie, nicht an das Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz, re-reduziert, also in seinen ursprünglichen vor der Lichteinstrahlung vorhandenen Zustand, zurückversetzt wird.
- 67. Verfahren nach den Ansprüchen 64 oder 65, dadurch gekennzeichnet, dass das nach Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entstandene reduzierte Elektron-Akzeptor-Molekül der photoinduzierbar redoxaktiven Einheit durch eine geeignete, freie, nicht an das Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz, re-oxidiert, also in seinen ursprünglichen vor der Lichteinstrahlung vorhandenen Zustand, zurückversetzt wird.
- 68. Verfahren zur elektrochemischen Detektion nach den Ansprüchen 62 oder 63, dadurch gekennzeichnet, dass die elektrochemische Detektion durch eine freie redoxaktive Substanz, durch die ein thermischer Ladungstransfer auf die redoxaktive Einheit stattfindet, ermöglicht wird.
- 69. Verfahren nach den Ansprüchen 67 oder 68, dadurch gekennzeichnet, dass die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz bei einem Potential φ selektiv oxidierbar und reduzierbar ist, wobei φ der Bedingung 2,0 V ≥ φ ≥ - 2,0 V, gemessen gegen Normalwasserstoffelektrode, genügt.
- 70. Verfahren nach den Ansprüchen 66 bis 69, dadurch gekennzeichnet, dass die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz ein freies Chinon, ein freier Hexacyanoferrat(II)-Komplex, ein freies Natriumascorbat, ein freier Ru(II)hexamin-Komplex oder ein freies redoxaktives Protein ist.
- 71. Verfahren nach Anspruch 70, dadurch gekennzeichnet, dass die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz ein freies Cytochrom ist.

VERTRAG ÜBER

EINTERNATIONALE ZUSAMN® 'ARBEIT AUF DEM GEBIET DES PATENTWESENS

Absender:

MIT DER INTERNATIONALEN VORLÄUFIGEN

PRÜFUNG BEAUFTRAGTE BEHÖRDE

An:

HARTWICH, Gerhard Nibelungenstrasse 10 D-80639 München **ALLEMAGNE**

MITTEILUNG ÜBER DIE ÜBERSENDUNG DES INTERNATIONALEN VORLÄUFIGEN PRÜFUNGSBERICHTS

(Regel 71.1 PCT)

Absendedatum

(Tag/Monat/Jahr)

21.03.2001

WICHTIGE MITTEILUNG

Aktenzeichen des Anmelders oder Anwalts

FRI006/00

Internationales Anmeldedatum (Tag/Monat/Jahr)

07/01/2000

Prioritätsdatum (Tag/Monat/Jahr)

18/01/1999

Anmelder

FRIZ BIOCHEM GMBH

Internationales Aktenzeichen

PCT/EP00/00084

- 1. Dem Anmelder wird mitgeteilt, daß ihm die mit der internationalen vorläufigen Prüfung beauftragte Behörde hiermit den zu der internationalen Anmeldung erstellten internationalen vorläufigen Prüfungsbericht, gegebenenfalls mit den dazugehörigen Anlagen, übermittelt.
- 2. Eine Kopie des Berichts wird gegebenenfalls mit den dazugehörigen Anlagen dem Internationalen Büro zur Weiterleitung an alle ausgewählten Ämter übermittelt.
- 3. Auf Wunsch eines ausgewählten Amts wird das Internationale Büro eine Übersetzung des Berichts (jedoch nicht der Anlagen) ins Englische anfertigen und diesem Amt übermitteln.

4. ERINNERUNG

Zum Eintritt in die nationale Phase hat der Anmelder vor jedem ausgewählten Amt innerhalb von 30 Monaten ab dem Prioritätsdatum (oder in manchen Ämtern noch später) bestimmte Handlungen (Einreichung von Übersetzungen und Entrichtung nationaler Gebühren) vorzunehmen (Artikel 39 (1)) (siehe auch die durch das Internationale Büro im Formblatt PCT/IB/301 übermittelte Information).

lst einem ausgewählten Amt eine Übersetzung der internationalen Anmeldung zu übermitteln, so muß diese Übersetzung auch Übersetzungen aller Anlagen zum internationalen vorläufigen Prüfungsbericht enthalten. Es ist Aufgabe des Anmelders, solche Übersetzungen anzufertigen und den betroffenen ausgewählten Ämtern direkt zuzuleiten.

Weitere Einzelheiten zu den maßgebenden Fristen und Erfordernissen der ausgewählten Ämter sind Band II des PCT-Leitfadens für Anmelder zu entnehmen.

Name und Postanschrift der mit der internationalen Prüfung beauftragten Behörde Europäisches Patentamt

Digiusto, M

D-80298 München

Tel. +49 89 2399 - 0 Tx: 523656 epmu d

Fax: +49 89 2399 - 4465

Tel. +49 89 2399-8162

Bevollmächtigter Bediensteter



VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM **GEBIET DES PATENTWESENS**

PCT

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

(Artikel 36 und Regel 70 PCT)

		(Artikel 36 und Reg	el 70 PC	1)
Aktenzeichen des	Anmelders oder Anwalts	WEITERES VORGEHEN	siehe Mittei vorläufigen	lung über die Übersendung des internationalen Prüfungsberichts (Formblatt PCT/IPEA/416)
Internationales Al	ttoozeichen	Internationales Anmeldedatum(7	ag/Monat/Jahr)	Prioritätsdatum (Tag/Monat/Tag)
PCT/EP00/00		07/01/2000		18/01/1999
		nationale Klassifikation und IPK		
C12Q1/68	(Priklassnikation (1714) 000.			
Anmelder				
FRIZ BIOCHE	EM GMBH			
Dieser inte Behörde e	ernationale vorläufige Pri rstellt und wird dem Ann	üfungsbericht wurde von der m nelder gemäß Artikel 36 überm	t der internati ttelt.	onalen vorläufigen Prüfung beauftragten
2. Dieser BE	RICHT umfaßt insgesam	nt 7 Blätter einschließlich diese	s Deckblatts.	
⊠ Außer und/o Behör	dem liegen dem Bericht	ANLAGEN bei; dabei handelt ändert wurden und diesem Bei richtigungen (siehe Regel 70.1	es sich um Blä	ätter mit Beschreibungen, Ansprüchen liegen, und/oder Blätter mit vor dieser itt 607 der Verwaltungsrichtlinien zum PCT).
	richt enthält Angaben zu			
	Grundlage des Berich			
II L) Prioritat) Voice Erstellung eine	s Gutachtens über Neuheit, erf	inderische Tä	tigkeit und gewerbliche Anwendbarkeit
_		· · · · · · · · · · · · · · · · · · ·		
V		ung nach Artikel 35(2) hinsichtli dbarkeit; Unterlagen und Erkläi	ch der Neuhe ungen zur Stü	it, der erfinderischen Tätigkeit und der itzung dieser Feststellung
VI 🗵	Bestimmte angeführte	e Unterlagen		
VII	Restimmte Mängel de	er internationalen Anmeldung		
_	Bestimmte Bernerkur	ngen zur internationalen Anmel	dung	
				D. data
Datum der Eini	eichung des Antrags	Date	ım der Fertigste	llung dieses Berichts
09/07/2000		21.0	3.2001	
Prüfung beauf	stanschrift der mit der interna tragten Behörde:	ationalen vorläufigen Bev	ollmächtigter Be	ediensteter (
	Suropäisches Patentamt 0-80298 München		vre, N	13 91 00 00 00 00 00 00 00 00 00 00 00 00 00
	rel. +49 89 2399 - 0 Tx: 523 rax: +49 89 2399 - 4465	Tai	Nr. +49 89 239	19 7363

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/00084

I.	Grundlage	des	Berichts
----	-----------	-----	----------

 Dieser Bericht wurde erstellt auf der Grundlage (Ersatzblätter, die dem Anmeldeamt auf eine Aufforderung nach Artikel 14 hin vorgelegt wurden, gelten im Rahmen dieses Berichts als "ursprünglich eingereicht" und sind ihm nicht beigefügt, weil sie keine Änderungen enthalten.): Beschreibung, Seiten:

2555111 4112 4113,				
1-61	ursprüngliche Fassung			
Patentansprüche, Nr.	:			
1-71	eingegangen am	14/11/2000	mit Schreiben vom	07/11/2000
Zeichnungen, Blätter	:			
1/6-6/6	ursprüngliche Fassung			

Sequenzprotokoll in der Beschreibung, Seiten:

- 1, eingereicht mit Schreiben vom 07.07.2000.
- Hinsichtlich der Sprache: Alle vorstehend genannten Bestandteile standen der Behörde in der Sprache, in der die internationale Anmeldung eingereicht worden ist, zur Verfügung oder wurden in dieser eingereicht, sofern unter diesem Punkt nichts anderes angegeben ist.

Die Bestandteile standen der Behörde in der Sprache: zur Verfügung bzw. wurden in dieser Sprache eingereicht; dabei handelt es sich um

	die Sprache der Übersetzung, die für die Zwecke der internationalen Recherche eingereicht worden ist (nach Regel 23.1(b)).
	die Voräffentlichungssprache der internationalen Anmeldung (nach Regel 48.3(b)).
	die Verörfermichungsoprache der übersetzung, die für die Zwecke der internationalen vorläufigen Prüfung eingereicht worden ist (nach Regel 55.2 und/oder 55.3).
Hin: inte	sichtlich der in der internationalen Anmeldung offenbarten Nucleotid- und/oder Aminosäuresequenz ist die rnationale vorläufige Prüfung auf der Grundlage des Sequenzprotokolls durchgeführt worden, das:
	in der internationalen Anmeldung in schriftlicher Form enthalten ist.
	zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.
Ø	bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.
Ø	bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.
Ø	Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.
×	Die Erklärung, daß die in computerlesbarer Form erfassten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/00084

4.	Auf	grund der Änderunger	n sind folgend	e U	nterlagen for	gefallen	:					
		Beschreibung, Ansprüche, Zeichnungen,	Seiten: Nr.: Blatt:									
5.		Dieser Bericht ist ohr angegebenen Gründ eingereichten Fassu	en nach Auffa	SSL	ung der Behör	de über	Änderung den Offe	gen erst enbarun	ellt worde gsgehalt	en, da di in der ur	iese aus rsprüngli	den ich
		(Auf Ersatzblätter, di beizufügen).	e solche Ände	erur	ngen enthaltei	n, ist unt	er Punkt	1 hinzu	weisen;si	e sind d	liesem B	ericht
6.	Etw	aige zusätzliche Bem	erkungen:									
٧.	Beg gew	ründete Feststellun verblichen Anwendb	arkeit; Unterl	l 3	5(2) hinsichtl en und Erklä	ich der ungen :	Neuheit, zur Stütz	, der erf zung die	inderiscl eser Fest	her Täti tstallung	igkeit ur a	nd der
1.	Fes	tstellung	,									
	Neu	heit (N)	Ja Ne		Ansprüche Ansprüche	1-71						
	Erfir	nderische Tätigkeit (E	T) Ja Ne		Ansprüche Ansprüche	1-71						
	Gev	verbliche Anwendbark	eit (GA) Ja No		Ansprüche Ansprüche	1-71						
2.	Unt siel	erlagen und Erklärung ne Beiblatt	gen									
۷I	. Bes	stimmte angeführte l	Interlagen									
1.	Bes	timmte veröffentlichte	Unterlagen (I	Яeς	jel 70.10)							

siehe Beiblatt

und / oder

2. Nicht-schriftliche Offenbarungen (Regel 70.9)

Zu Punkt I

Grundlage des Berichts

Das Sequenzprotokol, welches mit dem Schreiben vom 07.07.2000 eingereicht wurde, ist nicht Bestandteil der Anmeldung (Regel 13*ter*.1 f) PCT).

Zu Punkt V

Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung

1. Dokument D1 (US-A-5 770 369) offenbart Nukleinsäure-Oligomere, die durch kovalente Anbindung von redoxaktiven Verbindungen, wie zum Beispiel Übergangsmetallkomplexe, modifiziert sind (Abstract). Die Übergangsmetallkomplexe können unter anderem die Metalle Cd, Mg, Cu, Co, Pd, Zn, Fe, Ru, Rh, Os, Re, Pt, Sc, Ti, V, Cr, Mn, Ni, Mo, Tc, W und Ir enthalten (Spalte 7, Zeile 35 bis 48). Als Liganden der Metalle werden in D1 unter anderem Pyridin, Pyrazin, Bipyridin, Phenanthrolin, 9,10-Phenanthrenchinondiimin, Porphyrine und substituierte Derivate der Porphyrine (Spalte 7, Zeile 49 bis 62) genannt. Alternativ zu den Übergangsmetallkomplexen können auch andere organische Elektronendonoren bzw. -akzeptoren kovalent an die Nukleinsäuren gebunden werden (Spalte 8, Zeile 25 bis 27), zum Beispiel Riboflavin, Chinone, Porphyrine, ..., (Spalte 8, Zeile 27 bis 43).

Gemäß einer Ausführungsform von D1 werden die Nukleinsäuren an eine Elektrode gebunden (Spalte 8, Zeite 47 bis 50), wodurch ein Elektronentransport zwischen der Elektrode und der Nukleinsäure direkt über eine kovalente Bindung erfolgen kann (Spalte 9, Zeile 56 bis 58).

Somit stellt D1 ein System zur Verfügung, bei dem ein mit einem redoxaktiven organischen Molekül modifizertes Oligonukleotid an eine Elektrode gebunden ist (Spalte 11, Zeile 1 bis 12). Das Vorliegen einer doppelsträngigen DNA kann durch Vergleich der Elektronentransferraten von unhybridisierter Probe und

hybridisierter Probe erfolgen (Spalte 12, Zeile 44 bis 48). Zur Detektion des Elektronentransfers wird unter anderem die Amperometrie vorgeschlagen, was einen Vergleich der Stromstärke bei Vorliegen von hybridisierter bzw. unhybridisierter DNA ermöglicht (Spalte 27, Zeile 20 bis 47). Zur Initiierung der Elektronenübertragung kann Bestrahlung mit Licht angewendet werden (Spalte 23, Zeile 50 bis 53).

- Dokument D2 (WO-A-98 20162) offenbart Nukleinsäuren, die durch kovalente 1.1 Anbindung von redoxaktiven Verbindungen, wie zum Beispiel Übergangsmetallkomplexe, modifiziert sind (Seite 37, Zeile 22 und Seite 25, Zeile 27 bis 33). Als Liganden der Metalle werden unter anderem Pyridin, Pyrazin, Bipyridin, Phenanthrolin, ..., genannt (Seite 26, Zeile 14 bis 29). Alternativ zu den Übergangsmetallkomplexen können andere organische Elektronen-donoren bzw. -akzeptoren kovalent an die Nukleinsäuren gebunden werden (Seite 37, Zeile 29 bis 31), zum Beispiel Riboflavin, Chinone, Porphyrine, ... (Seite 37, Zeile 30 bis Seite 38, Zeile 5).
 - Die modifizierten Nukleinsäuren werden über ein leitfähiges Polymer an eine Elektrode gebunden (Anspruch 1), wodurch ein Elektronentransport zwischen der Elektrode und der Nukleinsäure erfolgen kann (Anspruch 9). Somit stellt D2 ein System zur Verfügung, bei dem ein mit einem redoxaktiven
 - organischen Molekül modifiziertes Oligonukleotid über ein leitfähiges Polymer an eine Elektrode gebunden ist (Anspruch 1 + 9). Das Vorliegen einer doppelsträngigen DNA kann durch erhöhten Elektronentransfer von der DNA auf die Elektrode detektiert werden (Seite 52, Zeile 21 bis 24). Zur Detektion des Elektronentransfers wird unter anderem die Amperometrie vorgeschlagen (Seite 56, Zeile 18 bis 25).
- 1.2 Dokument D3 (DE-A-42 16 696) offenbart ein Verfahren zur Durchführung von Assays, die unter anderem auf Wechselwirkungen zwischen DNA-Strängen beruhen. Die Detektion basiert zum Beispiel auf einer Messung der Stromstärke, wobei stabile redox-markierte Analytmoleküle Verwendung finden (Abstract). Dazu wird ein DNA-Strang mit einem stabilen Redox-System kovalent verbunden (Spalte 4, Zeile 6 bis 14). Als Redox-Systeme werden Ferrocenderivate, Ruthenium-komplexe, Hydrochinone, Hexacyanoferrat (II/III), Jod/Jodid oder ähnliche vorgeschlagen (z.B. Anspruch 22).

- 1.3 Weder eines der Dokumente D1 bis D3, noch eines der vorhandenen Dokumenten des Standes der Technik, offenbart oder legt ein durch eine redoxaktive Einheit modifiziertes Nukleinsäure-Oligomer nahe, bei dem die redoxaktive Einheit, wie von unabhängigen Anspruch 1 der vorliegenden Anmeldung gefordert, wenigstens ein Elektron-Donor-Molekül und wenigstens ein Elektron-Akzeptor-Molekül enthält, wobei Elektron-Donor-Molekül und Elektron-Akzeptor-Molekül nicht durch ein Nukleinsäure-Oligomer miteinander verbunden sind (siehe auch Seite 10, Zeile 18 bis 19, der Beschreibung).
- 1.4 Die Vorteile einer solches modifiziertes Nukleinsäure-Oligomers gegenüber den im Stand der Technik beschriebenen modifizierten Nukleinsäure-Oligomeren sind, unter anderen, dass die Übertragung einer Ladung aus dem angeregten Zustand nicht direkt auf die DNA, sondern direkt auf ein weiteres Molekül, das innerhalb der redoxaktiven Einheit vorhanden ist, geschieht. Erst von dem Akzeptor erfolgt die Übertragung der Ladung auf die DNA. Der Vorteil dieser aufeinanderfolgenden Übertragungen liegt in der Lebensdauer des Zustands der redoxaktiven Einheit nach der ersten Übertragung der Ladung. Diese ist wenigstens 103x, im Fall des photosynthetischen bakteriellen Reaktionszentrums (Seite 32) sogar 109x länger als die des ursprünglichen angeregten Zustandes des Donors. Um denselben Faktor erhöht sich auch die Wahrscheinlichkeit der Übertragung der Ladung auf die DNA, was drastische Auswirkungen auf die intensität des durch die DNA geleiteten Stroms hat.
- 1.5 Anspruch 1 ist daher neu und erfinderisch im Sinne von Artikeln 33(2) und 33(3) PCT.
- 1.6 Die abhängigen Ansprüche 2-27 definieren weitere spezifische Ausführungsformen des Anspruchs 1 und sind daher auch neu und erfinderisch im Sinne von Artikeln 33(2) und 33(3) PCT.
- Patentansprüche 28-36 definieren Verfahren zur Herstellung eines modifizierten 2. Nukleinsäure-Oligomers wie in Ansprüchen 1-27 beschrieben. Da diese modifizierten Nukleinsäure-Oligomers neu und erfinderisch sind, sind auch die in Ansprüchen 28-36 beschrieben Verfahren neu und erfinderisch im

Sinne von Artikeln 33(2) und 33(3) PCT.

- Dasselbe gilt auch für die in Patentansprüchen 37-57 definierten modifizierten leitfähigen Oberflächen, die dadurch gekennzeichnet sind, daß wenigstens ein modifiziertes Nukleinsäure-Oligomer wie in Ansprüchen 1-27 definiert, an eine solche leitfähige Oberfläche gebunden ist.
 - Patentansprüche 37-57 erfüllen daher die Erfordernisse des PCT in bezug auf Neuheit und erfinderische Tätigkeit im Sinne von Artikeln 33(2) und 33(3) PCT.
- 4. Analog dazu, sind die in Patentansprüchen 58-71 definierte Verfahren zur Herstellung solcher modifizierten leitfähigen Oberflächen ebenfalls neu und erfinderisch im Sinne von Artikeln 33(2) und 33(3) PCT.

<u>Zu Punkt VI</u> Bestimmte angeführte Unterlagen

Bestimmte veröffentlichte Unterlagen (Regel 70.10)

			Prioritätsdatum
Anmelde Nr. Patent Nr.	Veröffentlichungsdatum (Tag/Monat/Jahr)	Anmeldedatum (Tag/Monat/Jahr)	(zu Recht beansprucht) (Tag/Monat/Jahr)
WO-A-99 51778 WO-A-00 31101	14.10.1999 02.06.2000	07.04.1999 19.11.1999	08.04.1998 23.11.1998 29.04.1999

Bei Eintritt in die Regionalphase könnten obengenannte Dokumente für die Beurteilung der Neuheit relevant werden.

Die Gültigkeit des Prioritätsanspruch wurde nicht überprüft

Neue Patentansprüche 1 bis 71

- Durch kovalente Anbindung einer redoxaktiven Einheit modifiziertes Nukleinsäure-Oligomer, dadurch gekennzeichnet, dass die redoxaktive Einheit wenigstens ein Elektron-Donor-Molekül und wenigstens ein Elektron-Akzeptor-Molekül enthält, wobei Elektron-Donor-Molekül und Elektron-Akzeptor-Molekül nicht durch Nukleinsäure-Oligomere miteinander verbunden sind.
- 1. dadurch Anspruch Nukleinsäure-Oligomer nach Modifiziertes 2. gekennzeichnet, dass die redoxaktive Einheit wenigstens einen redoxaktiven, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptorverknüpften, Elektron-Donor-Molekül ein wenigstens Komplex enthält, wobei redoxaktiven Einheit und wenigstens ein Elektron-Akzeptor-Molekül der redoxaktiven Einheit durch eine oder mehrere Bindungen miteinander verbunden sind.
- 3. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 2, dadurch gekennzeichnet, dass es sich bei den Bindungen um kovalente Bindungen handelt.
- dadurch Nukleinsäure-Oligomer nach Anspruch 1. Modifiziertes 4. gekennzeichnet, dass die redoxaktive Einheit wenigstens einen redoxaktiven, verknüpften, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Elektron-Donor-Molekül Komplex enthält, wobei wenigstens ein redoxaktiven Einheit und wenigstens ein Elektron-Akzeptor-Molekül der redoxaktiven Einheit durch einen oder mehrere verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent verbunden sind.
- Modifiziertes Nukleinsäure-Oligomer nach Anspruch 4, dadurch gekennzeichnet, dass die verzweigten oder unverzweigten Molekülteile eine Kettenlänge von 1 - 20 Atomen aufweisen.
- Modifiziertes Nukleinsäure-Oligomer nach Anspruch 5, dadurch gekennzeichnet, dass die verzweigten oder unverzweigten Molekülteile eine Kettenlänge von 1 - 14 Atomen, aufweisen.

- Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit zusätzlich ein oder mehrere Makromoleküle umfaßt.
- 8. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei die redoxaktive Einheit das native oder modifizierte Reaktionszentrum von Photosynthese betreibenden Organismen ist.
- 9. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 8, wobei die redoxaktive Einheit das native oder modifizierte Reaktionszentrum von Photosynthese betreibenden Bakterien ist.
- Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass wenigstens eines der Elektron-Donor-Moleküle und Elektron-Akzeptor-Moleküle ein Farbstoff ist.
- Modifiziertes Nukleinsäure-Oligomer nach Anspruch 10, dadurch gekennzeichnet, dass es sich bei dem Farbstoff um ein Flavin, ein (Metallo-) Porphyrin, ein (Metallo-)Chlorophyll, ein (Metallo-)Bakteriochlorophyll oder ein Derivat dieser Farbstoffe handelt.
- Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass wenigstens eines der Elektron-Donor-Moleküle und Elektron-Akzeptor-Moleküle ein Nikotinsäureamid oder ein Chinon ist.
- 13. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 12, dadurch gekennzeichnet, dass es sich bei dem Chinon um ein Pyrrolo-Chinolin-Chinon (PQQ), ein 1,2-Benzochinon, ein 1,4-Benzochinon, ein 1,2-Naphtochinon, ein 9,10-Anthrachinon oder eines derer Derivate handelt.
- 14. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass wenigstens eines der Elektron-Donor-Moleküle und Elektron-Akzeptor-Moleküle ein Charge-Transfer-Komplex ist.
- 15. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 14, dadurch gekennzeichnet, dass der Charge-Transfer-Komplex ein Übergangsmetall-Komplex ist.

- 16. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 15, dadurch gekennzeichnet, dass der Charge-Transfer-Komplex ein Ru(II)-, ein Cr(III)-, ein Fe(II)-, ein Os(II)- oder ein Co(II)-Komplex ist.
- 17. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass das modifizierte Nukleinsäure-Oligomer sequenzspezifisch Einzelstrang-DNA, RNA und/oder PNA binden kann.
- 18. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 17, dadurch gekennzeichnet, dass das modifizierte Nukleinsäure-Oligomer ein Desoxyribonukleinsäure-, ein Ribonukleinsäure- oder ein Peptidnukleinsäure-Oligomer ist.
- 19. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit kovalent an eine der Phosphorsäure-Gruppen, an eine der Carbonsäure-Gruppen, an eine der Amin-Gruppen oder an einen Zucker des Nukleinsäure-Oligomer-Rückgrats gebunden ist.
- 20. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit kovalent an an eine Zucker-Hydroxy-Gruppe des Nukleinsäure-Oligomer-Rückgrats gebunden ist.
- 21. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 18, dadurch gekennzeichnet, dass die redoxaktive Einheit kovalent an eine Thiol-Gruppe, eine Hydroxy-Gruppe, eine Carbonsäure-Gruppe oder eine Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.
- 22. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 21, dadurch gekennzeichnet, dass die reaktive Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe der Base kovalent über einen verzweigten oder unverzweigten Molekülteil beliebiger Zusammensetzung und Kettenlänge an die Base gebunden ist, wobei die kürzeste durchgehende Verbindung zwischen der Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe und der Base ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen ist.

- 23. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 22, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen der Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe und der Base ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 14 Atomen ist.
- 24. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 19 bis 22, dadurch gekennzeichnet, dass die redoxaktive Einheit an ein Ende des Nukleinsäure-Oligomer-Rückgrats oder an eine endständige, modifizierte Base angebunden ist.
- 25. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die redoxaktive Einheit eine photoinduzierbar redoxaktive Einheit ist.
- 26. Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 24, dadurch gekennzeichnet, dass die redoxaktive Einheit eine chemisch induzierbar redoxaktive Einheit ist.
- 27. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass mehrere redoxaktive Einheiten an das Nukleinsäure-Oligomer angebunden sind.
- 28. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers wie in einem der vorhergehenden Ansprüche definiert, dadurch gekennzeichnet, dass eine redoxaktive Einheit kovalent an ein Nukleinsäure-Oligomer angebunden wird.
- 29. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 28, dadurch gekennzeichnet, dass die redoxaktive Einheit durch kovalente Anbindung von wenigstens einem Elektron-Donor-Molekül an ein Nukleinsäure-Oligomer angebunden wird.
- 30. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 28, dadurch gekennzeichnet, dass die redoxaktive Einheit durch kovalente Anbindung von wenigstens einem Elektron-Akzeptor-Molekül an ein Nukleinsäure-Oligomer angebunden wird.

- 31. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 28, dadurch gekennzeichnet, dass die redoxaktive Einheit durch kovalente Anbindung wenigstens eines Makromoleküls oder durch kovalente Anbindung wenigstens eines Proteins an ein Nukleinsäure-Oligomer angebunden wird.
- 32. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach den Ansprüchen 29 bis 31, dadurch gekennzeichnet, dass die redoxaktive Einheit durch Zugabe von wenigstens einem Bestandteil ausgewählt aus der Gruppe bestehend aus Elektron-Akzeptor-Moleküle, Elektron-Donor-Moleküle, Makromoleküle und Proteine vervollständigt wird.
- 33. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 28 bis 32, dadurch gekennzeichnet, dass das Nukleinsäure-Oligomer durch eine oder mehrere Amidbildungen mit Amin- oder mit Säure-Gruppen der red xaktiven Einheit, durch eine oder mehrere Esterbildungen mit Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit, durch Thioesterbildung mit Thio-Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit oder durch Kondensation einer oder mehrerer Amin-Gruppen des Nukleinsäure-Oligomers mit Aldehyd-Gruppen der redoxaktiven Einheit und anschließender Reduktion der entstandenen Kohlenstoff-Stickstoff-Doppelbindung an die redoxaktive Einheit gebunden wird.
- 34. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 28 bis 33, dadurch gekennzeichnet, dass wenigstens ein verzweigter oder unverzweigter Molekülteil beliebiger Zusammensetzung und Kettenlänge kovalent an die redoxaktive Einheit angebunden ist und die verzweigten oder unverzweigten Molekülteile eine reaktive Amin-, Hydroxy-, Thiol-, Säure- oder Aldehyd-Gruppe zur kovalenten Anbindung an ein Nukleinsäure-Oligomer aufweisen.
- 35. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 34, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen dem Nukleinsäure-Oligomer und der redoxaktiven Einheit ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen ist.
- 36. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 35, dadurch gekennzeichnet, dass die kürzeste durchgehende

Verbindung zwischen dem Nukleinsäure-Oligomer und der redoxaktiven Einheit ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 - 14 Atomen ist.

- 37. Modifizierte leitfähige Oberfläche, dadurch gekennzeichnet, dass wenigstens eine Art von modifiziertem Nukleinsäure-Oligomer gemäß einem der Ansprüche 1 bis 27 an eine leitfähige Oberfläche angebunden ist.
- 38. Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einem Metall oder einer Metallegierung besteht.
- 39. Modifizierte leitfähige Oberfläche nach Anspruch 38, dadurch gekennzeichnet, dass die Oberfläche aus einem Metall ausgewählt aus der Gruppe Platin, Palladium, Gold, Cadmium, Quecksilber, Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium und Mangan besteht.
- 40. Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einem Halbleiter besteht.
- 41. Modifizierte leitfähige Oberfläche nach Anspruch 38, dadurch gekennzeichnet, dass die Oberfläche aus einem Halbleiter ausgewählt aus der Gruppe Kohlenstoff, Silizium, Germanium und Zinn besteht.
- 42. Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einer binären Verbindung der Elemente der Gruppen 14 und 16, einer binären Verbindung der Elemente der Gruppen 13 und 15, einer binären Verbindung der Elemente der Gruppen 15 und 16, oder einer binären Verbindung der Elemente der Gruppen 11 und 17 besteht.
- 43. Modifizierte leitfähige Oberfläche nach Anspruch 42, dadurch gekennzeichnet, dass die Oberfläche aus einem Cu(I)-Halogenid oder einem Ag(I)-Halogenid besteht.
- Modifizierte leitfähige Oberfläche nach Anspruch 37, dadurch gekennzeichnet, dass die Oberfläche aus einer ternären Verbindung der Elemente der Gruppen 11, 13 und 16 oder einer ternären Verbindung Elemente der Gruppen 12, 13 und 16 besteht.

- 45. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 44, dadurch gekennzeichnet, dass die Anbindung der modifizierten Nukleinsäure-Oligomere an die leitfähige Oberfläche kovalent oder durch Chemi- oder Physisorption erfolgt.
- 46. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 45, dadurch gekennzeichnet, dass eine der Phosphorsäure-, Carbonsäure-, Amin- oder eine Zucker-Gruppe des Nukleinsäure-Oligomer-Rückgrats kovalent oder durch Chemi- oder Physisorption an die leitfähige Oberfläche angebunden ist.
- 47. Modifizierte leitfähige Oberfläche nach Anspruch 46, dadurch gekennzeichnet, dass eine Zucker-Hydroxy-Gruppe des Nukleinsäure-Oligomer-Rückgrats kovalent oder durch Chemi- oder Physisorption an die leitfähige Oberfläche angebunden ist.
- 48. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 45, dadurch gekennzeichnet, dass eine Thiol-Gruppe, eine Hydroxy-Gruppe, eine Carbonsäure-Gruppe oder eine Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers kovalent oder durch Chemi- oder Physisorption an die leitfähige Oberfläche angebunden ist.
- 49. Modifizierte leitfähige Oberfläche nach den Ansprüchen 46 bis 48, dadurch gekennzeichnet, dass das modifizierte Nukleinsäure-Oligomer über eine Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats oder über eine Gruppe einer endständigen, modifizierten Base an die leitfähige Oberfläche gebunden ist.
- 50. Modifizierte leitfähige Oberfläche nach den Ansprüchen 37 bis 49, dadurch gekennzeichnet, dass an die leitfähige Oberfläche verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent oder durch Chemi- oder Physisorption angebunden sind und die modifizierten Nukleinsäure-Oligomere kovalent an diese Molekülteile angebunden sind.
- 51. Modifizierte leitfähige Oberfläche nach Anspruch 50, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen der leitfähigen Oberfläche und dem Nukleinsäure-Oligomer ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen ist.

- 52. Modifizierte leitfähige Oberfläche nach Anspruch 51, dadurch gekennzeichnet, dass die kürzeste durchgehende Verbindung zwischen der leitfähigen Oberfläche und dem Nukleinsäure-Oligomer ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 12 Atomen, ist.
- 53. Modifizierte leitfähige Oberfläche nach den Ansprüchen 50 bis 52, dadurch gekennzeichnet, dass der verzweigte oder unverzweigte Molekülteil an eine Phosphorsäure-Gruppe, eine Carbonsäure-Gruppe, eine Amin-Gruppe oder eine Zucker-Gruppe des Nukleinsäure-Oligomer-Rückgrats oder eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.
- 54. Modifizierte leitfähige Oberfläche nach Anspruch 53, dadurch gekennzeichnet, dass der verzweigte oder unverzweigte Molekülteil an eine Zucker-Hydroxy-Gruppe des Nukleinsäure-Oligomer-Rückgrats angebunden ist.
- 55. Modifizierte leitfähige Oberfläche nach Anspruch 53 oder 54, dadurch gekennzeichnet, dass der verzweigte oder unverzweigte Molekülteil an eine Phosphorsäure-, Zucker-Hydroxy-, Carbonsäure- oder Amin-Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats oder an eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer endständigen, modifizierten Base gebunden ist.
- 56. Modifizierte leitfähige Oberfläche nach einem der Ansprüche 37 bis 55, dadurch gekennzeichnet, dass jeweils überwiegend eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.
- 57. Modifizierte leitfähige Oberfläche nach Anspruch 56, dadurch gekennzeichnet, dass jeweils ausschließlich eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.
- 58. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 37 bis 57 definiert, dadurch gekennzeichnet, dass wenigstens eine Art von modifizierten Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht wird.

- 59. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 37 bis 57 definiert, dadurch gekennzeichnet, dass wenigstens eine Art von Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht wird und anschließend eine Modifikation der Nukleinsäure-Oligomere durch ein Verfahren gemäß den Ansprüchen 28 bis 36 durchgeführt wird.
- 60. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach Anspruch 58 oder 59, dadurch gekennzeichnet, dass die Nukleinsäure-Oligomere oder die modifizierten Nukleinsäure-Oligomere mit dem dazu jeweils komplementären Nukleinsäure-Oligomerstrang hybridisiert werden und in Form des Doppelstranghybrids auf die leitfähige Oberfläche aufgebracht werden.
- 61. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 58 oder 59, dadurch gekennzeichnet, dass das Nukleinsäure-Oligomer oder das modifizierte Nukleinsäure-Oligomer in Gegenwart von weiteren chemischen Verbindungen, die ebenfalls an die leitfähige Oberfläche angebunden werden, auf die leitfähige Oberfläche aufgebracht wird.
- 62. Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybridisierungsereignissen, dadurch gekennzeichnet, dass wenigstens eine modifizierte leitfähige Oberfläche, wie in den Ansprüchen 37 bis 57 definiert, mit Nukleinsäure-Oligomeren in Kontakt gebracht wird und anschließend eine Detektion der elektrischen Kommunikation zwischen der redoxaktiven Einheit und der leitfähigen Oberfläche erfolgt.
- 63. Verfahren nach Anspruch 62, dadurch gekennzeichnet, dass die Detektion cyclovoltametrisch, amperometrisch oder durch Leitfähigkeitsmessung erfolgt.
- 64. Verfahren nach den Ansprüchen 62 oder 63, dadurch gekennzeichnet, dass die elektrochemische Detektion durch photoinduzierte Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktiven Einheit gestartet wird.
- 65. Verfahren nach Anspruch 64, dadurch gekennzeichnet, dass die Lichteinstrahlung zur photoinduzierten Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktiven Einheit auf einen Bereich der leitfähigen Oberfläche mit wenigstens einer Art von modifizierten Nukleinsäure-Oligomeren begrenzt wird.

- 66. Verfahren nach den Ansprüchen 64 oder 65, dadurch gekennzeichnet, dass das nach Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entstandene oxidierte Elektron-Donor-Molekül der photoinduzierbar redoxaktiven Einheit durch eine geeignete, freie, nicht an das Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz, re-reduziert, also in seinen ursprünglichen vor der Lichteinstrahlung vorhandenen Zustand, zurückversetzt wird.
- 67. Verfahren nach den Ansprüchen 64 oder 65, dadurch gekennzeichnet, dass das nach Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entstandene reduzierte Elektron-Akzeptor-Molekül der photoinduzierbar redoxaktiven Einheit durch eine geeignete, freie, nicht an das Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz, re-oxidiert, also in seinen ursprünglichen vor der Lichteinstrahlung vorhandenen Zustand, zurückversetzt wird.
- 68. Verfahren zur elektrochemischen Detektion nach den Ansprüchen 62 oder 63, dadurch gekennzeichnet, dass die elektrochemische Detektion durch eine freie redoxaktive Substanz, durch die ein thermischer Ladungstransfer auf die redoxaktive Einheit stattfindet, ermöglicht wird.
- 69. Verfahren nach den Ansprüchen 67 oder 68, dadurch gekennzeichnet, dass die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz bei einem Potential φ selektiv oxidierbar und reduzierbar ist, wobei φ der Bedingung 2,0 V ≥ φ ≥ 2,0 V, gemessen gegen Normalwasserstoffelektrode, genügt.
- 70. Verfahren nach den Ansprüchen 66 bis 69, dadurch gekennzeichnet, dass die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz ein freies Chinon, ein freier Hexacyanoferrat(II)-Komplex, ein freies Natriumascorbat, ein freier Ru(II)hexamin-Komplex oder ein freies redoxaktives Protein ist.
- 71. Verfahren nach Anspruch 70, dadurch gekennzeichnet, dass die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz ein freies Cytochrom ist.

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:		11) International Publication Number: WO 98/20162
C12Q 1/68	A2	43) International Publication Date: 14 May 1998 (14.05.98)
(21) International Application Number: PCT/US (22) International Filing Date: 5 November 1997 (Sup Francisco CA 04111-4187 (118)
(30) Priority Data: 08/743,798 5 November 1996 (05.11.96) 60/040,155 7 March 1997 (07.03.97) 08/873,597 12 June 1997 (12.06.97) 08/879,510 08/899,510 24 July 1997 (24.07.97) 08/911,085 14 August 1997 (14.08.97) 08/911,589 14 August 1997 (14.08.97) (71) Applicant (for all designated States except US): C MICRO SENSORS (US/US): 101 Waverly Drive, CA 91105 (US).	I I I IIINICA	LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN ML, MR, NE, SN, TD, TG).
(72) Inventors; and (75) Inventors/Applicants (for US only): KAYYEM [US/US]; 428 South Sierra Bonita Avenue, Pass 91106 (US). O'CONNOR, Stephen, D. [US/US). South El Molino #16, Pasadena, CA 91106 (US). Michael [IL/US]; 276 South El Molino #33, Pass 91101 (US). YU, Changjun [CN/US]; 400 Ra Drive #32, Pasadena, CA 91030 (US).	idena, (JS]; 42). GOZ! adena, (Without international search report and to be republishe upon receipt of that report.

(57) Abstruct

The invention relates to nucleic acids covalently coupled to electrodes via conductive oligomers. More particularly, the invention is directed to the site-selective modification of nucleic acids with electron transfer moieties and electrodes to produce a new class of biomaterials, and to methods of making and using them.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

			6	LS	Lesotho	SI	Slovenia
AL	Albania	ES	Spain	LT	Lithuania	SK	Slovakia
AΜ	Armenia	FI	Finland	LU	Luxembourg	SN	Senegal
AT	Austria	FR	France	LV	Latvia	SZ	Swaziland
ΑŪ	Australia	GA	Gabon	MC	Monaco	TD	Chad
ΑZ	Azerbaijan	GB	United Kingdom	MD	Republic of Moldova	TG	Togo
BA	Bosnia and Herzegovina	GE	Georgia	MG	Madagascar	ТJ	Tajikistan
BB	Barbados	GH	Ghana	MK	The former Yugoslav	TM	Turkmenistan
BE	Belgium	GN	Guinea	.****	Republic of Macedonia	TR	Turkey
BF	Burkina Faso	GR	Greece	ML	Mali	TT	Trinidad and Tobago
BG	Bulgana	HU	Hungary	MN	Mongolia	UA	Ukraine
BJ	Benin	1E	Ireland	MR	Mauritania	UG	Uganda
BR	Brazil	IL	Israel	MW	Malawi	US	United States of America
BY	Belarus	IS	Iceland	MX	Mexico	UZ	Uzbekistan
CA	Canada	ΙŤ	Italy	NE.	Niger	VN	Viet Nam
CF	Central African Republic	JР	Japan	NL.	Netherlands	YU	Yugoslavia
CG	Congo	KE	Kenya	NO.	Norway	zw	Zimbabwe
CH	Switzerland	KG	Kyrgyzstan	NZ.	New Zealand		
CI	Côte d'Ivoire	KP	Democratic People's	PL	Poland		
CM	Cameroon		Republic of Korea	PT	Portugal		
CN	China	KR	Republic of Korea	RO	Romania		
CU	Cuba	KZ.	Kazakstan	RU	Russian Federation		
CZ	Czech Republic	LC	Saint Lucia	SD	Sudan		
DE	Germany	1.1	Liechtenstein	SE.	Sweden		
DK	Denmark	LK	Sri Lanka	SG.	Singapore		
EE	Estonia	LR	1.ibena	30	Jingapore		

WO 98/20162 PCT/US97/20014 ·

ELECTRODES LINKED VIA CONDUCTIVE OLIGOMERS TO NUCLEIC ACIDS

FIELD OF THE INVENTION

The invention relates to nucleic acids covalently coupled to electrodes via conductive oligomers. More particularly, the invention is directed to the site-selective modification of nucleic acids with electron transfer moieties and electrodes to produce a new class of biomaterials, and to methods of making and using them.

5

10

BACKGROUND OF THE INVENTION

The detection of specific nucleic acids is an important tool for diagnostic medicine and molecular biology research. Gene probe assays currently play roles in identifying infectious organisms such as bacteria and viruses, in probing the expression of normal genes and identifying mutant genes such as oncogenes, in typing tissue for compatibility preceding tissue transplantation, in matching tissue or blood samples for forensic medicine, and for exploring homology among genes from different species.

Ideally, a gene probe assay should be sensitive, specific and easily automatable (for a review, see Nickerson, Current Opinion in Biotechnology 4:48-51 (1993)). The requirement for sensitivity (i.e. low detection limits) has been greatly alleviated by the development of the polymerase chain reaction (PCR) and other amplification technologies which allow researchers to amplify exponentially a specific nucleic acid sequence before analysis (for a review, see Abramson et al., Current Opinion in Biotechnology, 4:41-47 (1993)).

20

15

Specificity, in contrast, remains a problem in many currently available gene probe assays. The extent of molecular complementarity between probe and target defines the specificity of the interaction. Variations in the concentrations of probes, of targets and of salts in the hybridization medium, in the reaction temperature, and in the length of the probe may alter or influence the specificity of the probe/target interaction.

It may be possible under some limited circumstances to distinguish targets with perfect complementarity from targets with mismatches, although this is generally very difficult using traditional technology, since small variations in the reaction conditions will alter the hybridization. New experimental techniques for mismatch detection with standard probes include DNA ligation assays

30

25

where single point mismatches prevent ligation and probe digestion assays in which mismatches create sites for probe cleavage.

Finally, the automation of gene probe assays remains an area in which current technologies are lacking. Such assays generally rely on the hybridization of a labelled probe to a target sequence followed by the separation of the unhybridized free probe. This separation is generally achieved by gel electrophoresis or solid phase capture and washing of the target DNA, and is generally quite difficult to automate easily.

The time consuming nature of these separation steps has led to two distinct avenues of development.

One involves the development of high-speed, high-throughput automatable electrophoretic and other separation techniques. The other involves the development of non-separation homogeneous gene probe assays.

PCT applications WO 96/15971, PCT/US96/09769 and PCT/US97/09739 describe novel compositions comprising nucleic acids containing electron transfer moieties, including electrodes, which allow for novel detection methods of nucleic acid hybridization.

SUMMARY OF THE INVENTION

20

25

5

Accordingly, it is an object of the invention to provide for improved compositions and methods for the detection of nucleic acids.

In one aspect, the invention provides compositions comprising (a) a first electron transfer moiety comprising an electrode; (b) a first single stranded nucleic acid; (c) a second electron transfer moiety covalently attached to the first nucleic acid; and (d) a conductive oligomer covalently attached to both the electrode and the first nucleic acid.

In an additional aspect, the invention provides compositions comprising (a) a first electron transfer moiety comprising an electrode; (b) a first single stranded nucleic acid; (c) a conductive oligomer covalently attached to both the electrode and the first nucleic acid; and (d) a second electron transfer moiety covalently attached to a second single stranded nucleic acid.

35

30

In one aspect, the conductive oligomer has the formula:

$$\left(\left(\theta \right)_{g} D \right)_{e} \left(Y \right)_{m}$$

wherein

Y is an aromatic group;

5 n is an integer from 1 to 50;

g is either 1 or zero;

e is an integer from zero to 10; and

m is zero or 1;

wherein when g is 1, B-D is a conjugated bond; and

wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus.

In an additional aspect, the conductive oligomer has the formula:

15

25

30

wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene.

In a further aspect, the invention provides methods of detecting a target sequence in a nucleic acid sample. The method comprises applying a first input signal to a hybridization complex and detecting electron transfer. The hybridization complex comprises the target sequence, if present, and at least a first probe nucleic acid. The probe nucleic acid comprises a a covalently attached conductive oligomer. The conductive oligomer is also covalently attached to a first electron transfer moiety comprising an electrode. In addition, the hybridization complex has a covalently attached second electron transfer moiety.

In one aspect, the conductive oligomer has the formula:

$$\frac{-\left(-\left(8\right)_{g}-0\right)_{e}^{2}\left(-\left(8\right)_{g}\right)_{n}}{\left(-\left(8\right)_{g}\right)_{n}}$$

35

or

$$c$$
 c c

5 In one aspect, the first input signal comprises an AC component and a non-zero DC component.

In an additional aspect, the first input signal comprises an AC component at a first frequency and a non-zero DC component, and the method further comprises applying a second input signal comprising an AC component at at least a second frequency and a non-zero DC component.

10

In a further aspect, the first input signal comprises an AC component and a first non-zero DC component, and the method further comprises applying a second input signal comprising an AC component and a second non-zero DC component.

In an additional aspect, the first input signal comprises an AC component at a fist voltage amplitude and the method further comprises applying a second input signal comprising an AC component at a second voltage amplitude.

In an additional aspect, the invention provides methods of making the compositions of the invention.

The methodscomprise attaching a conductive oligomer to a nucleic acid, and attaching the conductive oligomer to said electrode. These steps may be done in any order.

In a further aspect, the invention provides compositions comprising a conductive oligomer covalently attached to a nucleoside, wherein said conductive oligomer has the formula: is selected from the group consisting of:

$$\frac{-\left(-\left(\theta\right)_{g}D\right)_{c}\left(-\left(\frac{1}{2}\right)_{g}D\right)_{g}}{\left(-\left(\frac{1}{2}\right)_{g}D\right)_{g}}$$

or

30

25

wherein

n is an integer from 1 to 50;

m is 0 or 1:

35 C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not alkane.

In an additional aspect, the invention provides compositions comprising (a) a solid support comprising a monolayer of passivation agent; (b) a nucleic acid comprising at least one nucleoside, wherein said nucleic acid is covalently attached to said solid support with a linker selected from the group selected from:

$$\frac{-\left(-\left(\theta\right)_{g}^{2}\right)_{c}^{2}\left(1-\left(\theta\right)_{g}^{2}\right)}{\left(1-\left(\theta\right)_{g}^{2}\right)_{c}^{2}\left(1-\left(\theta\right)_{g}^{2}\right)}$$

or

10

wherein

15 ris an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

20 G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not alkane

In an additional aspect, the invention provides compositions comprising (a) an electrode, (b) at least one metallocene; and (c) a conductive oligomer covalently attached to both said electrode and said metallocene, wherein said conductive oligomer is selected from the group consisting of:

25

or

$$\frac{-\left(-\left(B\right)_{g}^{2}\right)_{c}^{2}\left(-\left(B\right)_{g}^{2}\right)_{n}^{2}\left(-\left(B\right)_{g}^$$

30

35

In a further aspect, the invention provides peptide nucleic acids with at least one chemical substituent covalently attached to the α -carbon of a subunit of the peptide nucleic acid.

In an additional aspect, the invention provides peptide nucleic acids with at least one chemical substituent covalently attached to an internal subunit of the peptide nucleic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the synthetic scheme for a conductive oligomer covalently attached to a uridine nucleoside via an amide bond.

5

15

20

25

30

35

Figure 2 depicts the synthetic scheme for covalently attaching a conductive oligomer covalently attached to a uridine nucleoside via an amine bond.

Figure 3 depicts the synthetic scheme for a conductive oligomer covalently attached to a uridine nucleoside via the base.

Figure 4 depicts the synthetic scheme for a conductive oligomer covalently attached to a nucleoside via a phosphate of the ribose-phosphate backbone. The conductive oligomer is a phenyl-acetylene Structure 5 oligomer, although other oligomers may be used, and terminates in an ethyl pyridine protecting group, as described herein, for attachment to gold electrodes.

Figure 5 depicts the synthetic scheme for a conductive oligomer covalently attached to a nucleoside via a phosphate of the ribose-phosphate backbone, using an amide linkage and an ethylene linker, although other linkers may be used. The conductive oligomer is a phenyl-acetylene Structure 5 oligomer, although other oligomers may be used, and terminates in an ethyl pyridine protecting group, as described herein, for attachment to gold electrodes.

Figure 6 depicts the synthetic scheme for a conductive polymer containing an aromatic group with a substitution group. The conductive oligomer is a phenyl-acetylene Structure 5 oligomer with a single methyl R group on each phenyl ring, although other oligomers may be used, and terminates in an ethyl pyridine protecting group, as described herein, for attachment to gold electrodes.

Figure 7 depicts the synthetic scheme for the synthesis of a metallocene, in this case ferrocene, linked via a conductive oligomer to an electrode. The conductive oligomer is a phenyl-acetylene Structure 5 oligomer, although other oligomers may be used, and terminates in an ethyl pyridine protecting group, as described herein, for attachment to gold electrodes.

Figure 8 depicts a model compound, ferrocene attached to a C_{16} alkane molecule (insulator-1), at 200 mV AC amplitude and frequencies of 1, 5 and 100 Hz. The sample responds at all three frequencies, with higher currents resulting from higher frequencies.

5

10

25

30

35

Figures 9A and 9B depict the response with varying frequency. Figure 9A shows overlaid voltammograms of an electrode coated with a ferrocene-conductive oligomer model complex (wire-2). Four excitation frequencies were applied, 10 Hz, 100 Hz, 1 kHz and 10 kHz, all at 25 mV overpotential. Again, current increases with frequency. Figure 9B shows overlaid voltammograms of electrodes coated with either ssDNA or dsDNA. ssDNA was run at 1 Hz and 10 Hz at 100 mV overpotential (bottom two lines). dsDNA was run at 1, 10, 50 and 100 Hz at 10 mV overpotential (top four lines). Note that the scales between Figure 8 and Figures 9A and 9B are different.

Figure 10 depicts the frequency response of these systems. The peak currents at a number of frequencies are determined and plotted. Sample 3 (filled triangles) responds to increasing frequencies through 10 kHz (system limit), while samples 1 (open circles) and 2 (filled circles) lose their responses at between 20 and 200 Hz. This data was not normalized to the increase in current associated with increasing frequency.

15 Figure 11 depicts the frequency responses of ssDNA (open circles; sample 5) and dsDNA (filled circles; sample 6) at 25 mV overpotential. The current has been normalized. The curves are not a fit to the data; rather, these are models of RC circuits, illustrating that the data can be fit to such curves, and that the system is in fact mimic standard RC circuits. The top curve was modeled using a 500 ohm resistor and a 0.001 farad capacitor. The bottom curve was modeled using a 20 ohm resistor and a 0.002 farad capacitor.

Figure 12 shows that increasing the overpotential will increase the output current.

Figures 13A and 13B illustrate that the overpotential and frequency can be tuned to increase the selectivity and sensitivity, using Sample 1

Figure 14 shows that ferrocene added to the solution (Sample 7; open circles) has a frequency response related to diffusion that is easily distinguishable from attached ferrocene (Sample 3; filled circles).

Figures 15A and 15B shows the phase shift that results with different samples. Figure 15A uses two experiments of Sample 1, Sample 3 and Sample 4. Figure 15B uses Sample 5 and Sample 6.

Figure 16 depicts the synthetic scheme for a conductive oligomer covalently attached to a uridine nucleoside via an amine bond, with a CH2 group as a Z linker. Compound C4 can be extended as outlined herein and in Figure 1

Figures 17A, 17B, 17C, 17D, 17E, 17F and 17G depict other conductive oligomers, attached either through the base (A-D) or through the ribose of the backbone (E-G), which have been synthesized using the techniques outlined herein. Figure 17H depicts a conductive oligomer attached to a ferrocene. As will be appreciated by those in the art, the compounds are shown as containing CPG groups, phosphoramidite groups, or neither; however, they may all be made as any of these.

Figure 18 depicts a synthetic scheme for a four unit conductive oligomer attached to the base.

Figure 19 depicts a synthetic scheme for a four unit conductive oligomer attached to the base.

10

5

Figure 20 depicts the use of a trimethylsilylethyl protecting group in synthesizing a five unit wire attached via the base.

Figure 21 depicts the use of a trimethylsilylethyl protecting group in synthesizing a five unit wire attached via the ribose.

Figures 22A and 22B depict simulations based on traditional electrochemical theory (Figure 22B) and the simulation model developed herein (Figure 22A).

Figures 23A and 23B depict experimental data plotted with theoretical model, showing good correlation. Fc-wire of Example 7 was used as 10 Hz (Figure 23A) and 100 Hz (Figure 23B).

Figure 24 depicts the synthetic scheme for protecting and derivatizing adenine for incorporation into PNA.

25

30

Figure 25 depicts the synthetic scheme for protecting and derivatizing cytosine for incorporation into PNA.

Figure 26 depicts the synthetic scheme for protecting and derivatizing guanine for incorporation into PNA.

Figure 27 depicts the synthetic scheme for protecting and derivatizing thymine for incorporation into PNA.

Figures 28A, 28B, 28C, 28D and 28E. Figure 28A depicts the synthetic scheme for making PNA monomeric subunits. Figures 28B-28E depict the PNA monomers.

5

15

20

25

30

35

Figure 29 depicts the synthetic scheme for a PNA monomeric subunit with a ferrocene covalently attached to a uracil base, for incorporation into a growing PNA.

Figure 30 depicts the synthetic scheme for a three unit conductive oligomer covalently attached to a base of a PNA monomeric subunit.

Figure 31 depicts the synthetic scheme for a three unit conductive oligomer covalently attached to the backbone of a PNA monomeric subunit.

Figure 32 depicts the synthetic scheme for a ferrocene covalently attached to the backbone of a PNA monomeric subunit.

DETAILED DESCRIPTION OF THE INVENTION

The present invention capitalizes on the previous discovery that electron transfer apparently proceeds through the stacked π-orbitals of the heterocyclic bases of double stranded (hybridized) nucleic acid ("the π-way"). This finding allows the use of nucleic acids containing electron transfer moieties to be used as nucleic acid probes. See PCT publication WO 95/15971, hereby incorporated by reference in its entirety, and cited references. This publication describes the site-selective modification of nucleic acids with redox active moieties, i.e. electron donor and acceptor moieties, which allow the long-distance electron transfer through a double stranded nucleic acid. In general, electron transfer between electron donors and acceptors does not occur at an appreciable rate when the nucleic acid is single stranded, nor does it occur appreciably unless nucleotide base pairing exists in the double stranded sequence between the electron donor and acceptor in the double helical structure. Thus, PCT publicationWO 95/15971 and the present invention are directed to the use of nucleic acids with electron transfer moieties, including electrodes, as probes for the detection of target sequences within a sample.

In one embodiment, the present invention provides for novel gene probes, which are useful in molecular biology and diagnostic medicine. In this embodiment, single stranded nucleic acids having a predetermined sequence and covalently attached electron transfer moieties, including an electrode, are synthesized. The sequence is selected based upon a known target sequence, such that if hybridization to a complementary target sequence occurs in the region between the electron donor and the electron acceptor, electron transfer proceeds at an appreciable and detectable rate. Thus, the invention has broad general use, as a new form of labelled gene probe. In addition, the probes of the

present invention allow detection of target sequences without the removal of unhybridized probe. Thus, the invention is uniquely suited to automated gene probe assays or field testing.

The present invention provides improved compositions comprising nucleic acids covalently attached via conductive oligomers to an electrode, of a general structure depicted below in Structure 1:

Structure 1

10

15

20

5

In Structure 1, the hatched marks on the left represent an electrode. X is a conductive oligomer as defined herein. F, is a linkage that allows the covalent attachment of the electrode and the conductive oligomer, including bonds, atoms or linkers such as is described herein, for example as "A", defined below. F₂ is a linkage that allows the covalent attachment of the concuctive oligomer to the nucleic acid, and may be a bond, an atom or a linkage as is herein described. F2 may be part of the conductive oligomer, part of the nucleic acid, or exogeneous to both, for example, as defined herein for "Z".

By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai 25 et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. 30 Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger 35 et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994);

5

10

15

20

-11-

Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of electron transfer moieties, or to increase the stability and half-life of such molecules in physiological environments.

As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; for example, at the site of conductive oligomer or electron transfer moiety attachment, an analog structure may be used. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (Tm) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4°C drop in Tm for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9°C. This allows for better detection of mismatches. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration. This is particularly advantageous in the systems of the present invention, as a reduced salt hybridization solution has a lower Faradaic current than a physiological salt solution (in the range of 150 mM).

30

35

25

The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. As used herein, the term "nucleoside" includes nucleotides and nucleoside and nucleotide analogs, and modified nucleosides such as amino

PCT/US97/20014 ~

modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

The nucleosides and nucleic acids are covalently attached to a conductive oligomer. By "conductive oligomer" herein is meant a substantially conducting oligomer, preferably linear, some embodiments of which are referred to in the literature as "molecular wires". By "substantially conducting" herein is meant that the rate of electron transfer through the conductive oligomer is faster than the rate of electron transfer through single stranded nucleic acid, such that the conductive oligomer is not the rate limiting step in the detection of hybridization, although as noted below, systems which use spacers that are the rate limiting step are also acceptable. Stated differently, the resistance of the conductive oligomer is less than that of the nucleic acid. Preferably, the rate of electron transfer through the conductive oligomer is faster than the rate of electron transfer through double stranded nucleic acid, i.e. through the stacked π-orbitals of the double helix. Generally, the conductive oligomer has substantially overlapping π-orbitals, i.e. conjugated π-orbitals, as between the monomeric units of the conductive oligomer, although the conductive oligomer may also contain one or more sigma (σ) bonds. Additionally, a conductive oligomer may be defined functionally by its ability to inject or receive electrons into or from an attached nucleic acid. Furthermore, the conductive oligomer is more conductive than the insulators as defined herein.

20

25

15

5

10

In a preferred embodiment, the conductive oligomers have a conductivity, S, of from between about 10^{-6} to about $10^{4} \ \Omega^{-1} \text{cm}^{-1}$, with from about 10^{-5} to about $10^{3} \ \Omega^{-1} \text{cm}^{-1}$ being preferred, with these S values being calculated for molecules ranging from about 20Å to about 200Å. As described below, insulators have a conductivity S of about $10^{-7} \ \Omega^{-1} \text{cm}^{-1}$ or lower, with less than about $10^{-8} \ \Omega^{-1} \text{cm}^{-1}$ being preferred. See generally Gardner et al., Sensors and Actuators A 51 (1995) 57-66, incorporated herein by reference.

30

Desired characteristics of a conductive oligomer include high conductivity, sufficient solubility in organic solvents and/or water for synthesis and use of the compositions of the invention, and preferably chemical resistance to reactions that occur i) during nucleic acid synthesis (such that nucleosides containing the conductive oligomers may be added to a nucleic acid synthesizer during the synthesis of the compositions of the invention), ii) during the attachment of the conductive oligomer to an electrode, or iii) during hybridization assays.

35

The oligomers of the invention comprise at least two monomeric subunits, as described herein. As is described more fully below, oligomers include homo- and hetero-oligomers, and include polymers.

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 2:

Structure 2

$$\frac{-\left(-\left(\theta\right)_{g}\circ\right)_{c}^{2}\left(-\left(\theta\right)_{g}\circ\right)_{c}^{2}\left(-\left(\theta\right)_{g}\circ(-\left(\theta\right)_{g}\circ\left(-\left(\theta\right)_{g}\circ\left(-\left(\theta\right)_{g}\circ\left(-\left(\theta\right)_{g}\circ\left(-\left(\theta\right)_{g}\circ(-$$

5

10

15

20

As will be understood by those in the art, all of the structures depicted herein may have additional atoms or structures; i.e. the conductive oligomer of Structure 2 may be attached to electron transfer moieties, such as electrodes, transition metal complexes, organic electron transfer moieties, and metallocenes, and to nucleic acids, or to several of these. Unless otherwise noted, the conductive oligomers depicted herein will be attached at the left side to an electrode; that is, as depicted in Structure 2, the left "Y" is connected to the electrode as described herein and the right "Y", if present, is attached to the nucleic acid, either directly or through the use of a linker, as is described herein.

In this embodiment, Y is an aromatic group in so an integer from 1 to 50, g is either 1 or zero, e is an integer from zero to 10, and m is zero or 1. When g is 1, B-D is a conjugated bond, preferably selected from acetylene, alkene, substituted alkene, amide, azo, -C=N- (including -N=C-, -CR=N- and -N=CR-), -Si=Si-, and -Si=C- (including -C=Si-, -Si=CR- and -CR=Si-). When g is zero, e is preferably 1, D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus. Thus, suitable heteroatom moieties include, but are not limited to, -NH and -NR, wherein R is as defined herein; substituted sulfur; sulfonyl (-SO₂-) sulfoxide (-SO-); phosphine oxide (-PO- and -RPO-); and thiophosphine (-PS- and -RPS-). However, when the conductive oligomer is to be attached to a gold electrode, as outlined below, sulfur derivatives are not preferred.

30

25

By "aromatic group" or grammatical equivalents herein is meant an aromatic monocyclic or polycyclic hydrocarbon moiety generally containing 5 to 14 carbon atoms (although larger polycyclic rings structures may be made) and any carbocylic ketone or thioketone derivative thereof, wherein the carbon atom with the free valence is a member of an aromatic ring. Aromatic groups include arylene groups and aromatic groups with more than two atoms removed. For the purposes of this application aromatic includes heterocycle. "Heterocycle" or "heteroaryl" means an aromatic group wherein 1 to 5 of the indicated carbon atoms are replaced by a heteroatom chosen from nitrogen, oxygen, sulfur, phosphorus, boron and silicon wherein the atom with the free valence is a member of an aromatic ring, and any heterocyclic ketone and thioketone derivative thereof. Thus, heterocycle includes thienyl, furyl, pyrrolyl, pyrimidinyl, oxalyl, indolyl, purinyl, quinolyl, isoquinolyl, thiazolyl, imidozyl, etc.

35

10

25

30

35

Importantly, the Y aromatic groups of the conductive oligomer may be different, i.e. the conductive oligomer may be a heterooligomer. That is, a conductive oligomer may comprise a oligomer of a single type of Y groups, or of multiple types of Y groups. Thus, in a preferred embodiment, when a barrier monolayer is used as is described below, one or more types of Y groups are used in the conductive oligomer within the monolayer with a second type(s) of Y group used above the monolayer level. Thus, as is described herein, the conductive oligomer may comprise Y groups that have good packing efficiency within the monolayer at the electrode surface, and a second type(s) of Y groups with greater flexibility and hydrophilicity above the monolayer level to facilitate nucleic acid hybridization. For example, unsubstituted benzyl rings may comprise the Y rings for monolayer packing, and substituted benzyl rings may be used above the monolayer. Alternatively, heterocytic rings, either substituted or unsubstituted, may be used above the monolayer. Additionally, in one embodiment, heterooligomers are used even when the conductive oligomer does not extend out of the monolayer.

The aromatic group may be substituted with a substitution group, generally depicted herein as R. R groups may be added as necessary to affect the packing of the conductive oligomers, i.e. when the nucleic acids attached to the conductive oligomers form a monolayer on the electrode, R groups may be used to alter the association of the oligomers in the monolayer. R groups may also be added to 1) alter the solubility of the oligomer or of compositions containing the oligomers; 2) alter the conjugation or electrochemical potential of the system; and 3) alter the charge or characteristics at the surface of the monolayer.

In a preferred embodiment, when the conductive oligomer is greater than three subunits, R groups are preferred to increase solubility when solution synthesis is done. However, the R groups, and their positions, are chosen to minimally effect the packing of the conductive oligomers on a surface, particularly within a monolayer, as described below. In general, only small R groups are used within the monolayer, with larger R groups generally above the surface of the monolayer. Thus for example the attachment of methyl groups to the portion of the conductive oligomer within the monolayer to increase solubility is preferred, with attachment of longer alkoxy groups, for example, C3 to C10, is preferably done above the monolayer surface. In general, for the systems described herein, this generally means that attachment of sterically significant R groups is not done on any of the first two or three oligomer subunits, depending on the length of the insulator molecules.

Suitable R groups include, but are not limited to, hydrogen, alkyl, alcohol, aromatic, amino, amido, nitro, ethers, esters, aldehydes, sulfonyl, silicon moieties, halogens, sulfur containing moieties, phosphorus containing moieties, and ethylene glycols. In the structures depicted herein, R is

hydrogen when the position is unsubstituted. It should be noted that some positions may allow two substitution groups, R and R', in which case the R and R' groups may be either the same or different.

By "alkyl group" or grammatical equivalents herein is meant a straight or branched chain alkyl group, with straight chain alkyl groups being preferred. If branched, it may be branched at one or more positions, and unless specified, at any position. The alkyl group may range from about 1 to about 30 carbon atoms (C1 -C30), with a preferred embodiment utilizing from about 1 to about 20 carbon atoms (C1 -C20), with about C1 through about C12 to about C15 being preferred, and C1 to C5 being particularly preferred, although in some embodiments the alkyl group may be much larger. Also included within the definition of an alkyl group are cycloalkyl groups such as C5 and C6 rings, and heterocyclic rings with nitrogen, oxygen, sulfur or phosphorus. Alkyl also includes heteroalkyl, with heteroatoms of sulfur, oxygen, nitrogen, and silicone being preferred. Alkyl includes substituted alkyl groups. By "substituted alkyl group" herein is meant an alkyl group further comprising one or more substitution moieties "R", as defined above.

15

10

5

By "amino groups" or grammatical equivalents herein is meant -NH $_2$, -NHR and -NR $_2$ groups, with R being as defined herein.

By "nitro group" herein is meant an -NO, group.

20

25

By "sulfur containing moieties" herein is meant compounds containing sulfur atoms, including but not limited to, thia-, thio- and sulfo- compounds, thiols (-SH and -SR), and sulfides (-RSR-). By "phosphorus containing moieties" herein is meant compounds containing phosphorus, including, but not limited to, phosphines and phosphates. By "silicon containing moieties" herein is meant compounds containing silicon.

By "ether" herein is meant an -O-R group. Preferred ethers include alkoxy groups, with -O- $(CH_2)_2CH_3$ and -O- $(CH_2)_4CH_3$ being preferred.

30 By "ester" herein is meant a -COOR group.

By "halogen" herein is meant bromine, iodine, chlorine, or fluorine. Preferred substituted alkyls are partially or fully halogenated alkyls such as CF₃, etc.

35 By "aldehyde" herein is meant -RCOH groups

By "alcohol" herein is meant -OH groups, and alkyl alcohols -ROH.

By "amido" herein is meant -RCONH- or RCONR- groups.

- By "ethylene glycol" or "(poly)ethylene glycol" herein is meant a -(O-CH₂-CH₂)_n- group, although each carbon atom of the ethylene group may also be singly or doubly substituted, i.e. -(O-CR₂-CR₂)_n-, with R as described above. Ethylene glycol derivatives with other heteroatoms in place of oxygen (i.e. -(N-CH₂-CH₂)_n- or -(S-CH₂-CH₂)_n-, or with substitution groups) are also preferred.
- Preferred substitution groups include, but are not limited to, methyl, ethyl, propyl, alkoxy groups such as -O-(CH₂)₂CH₃ and -O-(CH₂)₄CH₃ and ethylene glycol and derivatives thereof.

Preferred aromatic groups include, but are not limited to, phenyl, naphthyl, naphthalene, anthracene, phenanthroline, pyrole, pyridine, thiophene, porphyrins, and substituted derivatives of each of these, included fused ring derivatives.

In the conductive oligomers depicted herein, when g is 1, B-D is a bond linking two atoms or chemical moieties. In a preferred embodiment, B-D is a conjugated bond, containing overlapping or conjugated π -orbitals.

20

25

30

15

Preferred B-D bonds are selected from acetylene (-C=C-, also called alkyne or ethyne), alkene (-CH=CH-, also called ethylene), substituted alkene (-CR=CR-, -CH=CR- and -CR=CH-), amide (-NH-CO- and -NR-CO- or -CO-NH- and -CO-NR-), azo (-N=N-), esters and thioesters (-CO-O-, -O-CO-, -CS-O- and -O-CS-) and other conjugated bonds such as (-CH=N-, -CR=N-, -N=CH- and -N=CR-), (-SiH=SiH-, -SiR=SiH-, -SiR=SiH-, and -SiR=SiR-), (-SiH=CH-, -SiR=CH-, -SiH=CR-, -SiR=CR-, -CH=SiH-, -CR=SiH-, -CH=SiR-, and -CR=SiR-). Particularly preferred B-D bonds are acetylene, alkene, amide, and substituted derivatives of these three, and azo. Especially preferred B-D bonds are acetylene, alkene and amide. The oligomer components attached to double bonds may be in the trans or cis conformation, or mixtures. Thus, either B or D may include carbon, nitrogen or silicon. The substitution groups are as defined as above for R.

When g=0 in the Structure 2 conductive oligomer, e is preferably 1 and the D moiety may be carbonyl or a heteroatom moiety as defined above.

As above for the Y rings, within any single conductive oligomer, the B-D bonds (or D moieties, when g=0) may be all the same, or at least one may be different. For example, when m is zero, the terminal

10

15

20

25

30

35



-17-

B-D bond may be an amide bond, and the rest of the B-D bonds may be acetylene bonds. Generally, when amide bonds are present, as few amide bonds as possible are preferable, but in some embodiments all the B-D bonds are amide bonds. Thus, as outlined above for the Y rings, one type of B-D bond may be present in the conductive oligomer within a monolayer as described below, and another type above the monolayer level, to give greater flexibility for nucleic acid hybridization.

In the structures depicted herein, n is an integer from 1 to 50, although longer oligomers may also be used (see for example Schumm et al., Angew. Chem. Int. Ed. Engl. 1994 33(13):1360). Without being bound by theory, it appears that for efficient hybridization of nucleic acids on a surface, the hybridization should occur at a distance from the surface, i.e. the kinetics of hybridization increase as a function of the distance from the surface, particularly for long oligonucleotides of 200 to 300 basepairs. Accordingly, the length of the conductive oligomer is such that the closest nucleotide of the nucleic acid is positioned from about 6Å to about 100Å (although distances of up to 500Å may be used) from the electrode surface, with from about 15Å to about 60Å being preferred and from about 25Å to about 60Å also being preferred. Accordingly, n will depend on the size of the aromatic group, but generally will be from about 1 to about 20, with from about 2 to about 15 being preferred and from about 3 to about 10 being especially preferred.

In the structures depicted herein, m is either 0 or 1. That is, when m is 0, the conductive oligomer may terminate in the B-D bond or D moiety, i.e. the D atom is attached to the nucleic acid either directly or via a linker. In some embodiments, for example when the conductive oligomer is attached to a phosphate of the ribose-phosphate backbone of a nucleic acid, there may be additional atoms, such as a linker, attached between the conductive oligomer and the nucleic acid. Additionally, as outlined below, the D atom may be the nitrogen atom of the amino-modified ribose. Alternatively, when m is 1, the conductive oligomer may terminate in Y, an aromatic group, i.e. the aromatic group is attached to the nucleic acid or linker.

As will be appreciated by those in the art, a large number of possible conductive oligomers may be utilized. These include conductive oligomers falling within the Structure 2 and Structure 9 formulas, as well as other conductive oligomers, as are generally known in the art, including for example, compounds comprising fused aromatic rings or Teflon®-like oligomers, such as -(CF₂)_n-, -(CHF)_n- and -(CFR)_n-. See for example, Schumm et al., angew. Chem. Intl. Ed. Engl. 33:1361 (1994);Grosshenny et al., Platinum Metals Rev. 40(1):26-35 (1996); Tour. Chem. Rev. 96.537-553 (1996); Hsung et al., Organometallics 14:4808-4815 (1995; and references cited therein, all of which are expressly incorporated by reference.

Particularly preferred conductive oligomers of this embodiment are depicted below:

Structure 3

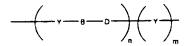
$$\frac{-\left(-\frac{1}{2}\left(-\frac{1}{2}-\frac{1}{2}\right)^{2}-\frac{1}{2}\left(-\frac{1}$$

5

10

Structure 3 is Structure 2 when g is 1. Preferred embodiments of Structure 3 include: e is zero, Y is pyrole or substituted pyrole; e is zero, Y is thiophene or substituted thiophene; e is zero, Y is furan or substituted furan; e is zero, Y is phenyl or substituted phenyl; e is zero, Y is pyridine or substituted pyridine; e is 1, B-D is acetylene and Y is phenyl or substituted phenyl (see Structure 5 below). A preferred embodiment of Structure 3 is also when e is one, depicted as Structure 4 below:

Structure 4



15

Preferred embodiments of Structure 4 are: Y is phenyl or substituted phenyl and B-D is azo; Y is phenyl or substituted phenyl and B-D is acetylene; Y is phenyl or substituted phenyl and B-D is alkene; Y is pyridine or substituted pyridine and B-D is acetylene; Y is thiophene or substituted thiophene and B-D is acetylene; Y is furan or substituted furan and B-D is acetylene; Y is thiophene or furan (or substituted thiophene or furan) and B-D are alternating alkene and acetylene bonds.

20

Most of the structures depicted herein utilize a Structure 4 conductive oligomer. However, any Structure 4 oligomers may be substituted with a Structure 2, 3 or 9 oligomer, or other conducting oligomer, and the use of such Structure 4 depiction is not meant to limit the scope of the invention.

25

Particularly preferred embodiments of Structure 4 include Structures 5, 6, 7 and 8, depicted below:

Structure 5

30

Particularly preferred embodiments of Structure 5 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen; and the use of R groups to increase solubility.

Structure 6

When the B-D bond is an amide bond, as in Structure 6, the conductive oligomers are pseudopeptide oligomers. Although the amide bond in Structure 6 is depicted with the carbonyl to the left, i.e. - CONH-, the reverse may also be used, i.e. -NHCO-. Particularly preferred embodiments of Structure 6 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen (in this embodiment, the terminal nitrogen (the D atom) may be the nitrogen of the amino-modified ribose); and the use of R groups to increase solubility.

Structure 7

Preferred embodiments of Structure 7 include the first n is two, second n is one, m is zero, and all R groups are hydrogen, or the use of R groups to increase solubility.

Structure 8

15

20

5

10



Preferred embodiments of Structure 8 include: the first n is three, the second n is from 1-3, with m being either 0 or 1, and the use of R groups to increase solubility.

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 9:

Structure 9

25

30

35

In this embodiment, C are carbon atoms, n is an integer from 1 to 50, m is 0 or 1, J is a heteroatom selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur, carbonyl or sulfoxide, and G is a bond selected from alkane, alkene or acetylene, such that together with the two carbon atoms the C-G-C group is an alkene (-CH=CH-), substituted alkene (-CR=CR-) or mixtures thereof (-CH=CR- or -CR=CH-), acetylene (-C=C-), or alkane (-CR2-CR2-, with R being either hydrogen or a substitution group as described herein). The G bond of each subunit may be the same or different than the G bonds of other subunits; that is, alternating oligomers of alkene and acetylene bonds could be used, etc. However, when G is an alkane bond, the number of alkane bonds in the oligomer should be kept to a minimum, with about six or less sigma bonds per conductive oligomer being preferred. Alkene bonds are preferred, and are generally depicted herein, although alkane and

-20-

acetylene bonds may be substituted in any structure or embodiment described herein as will be appreciated by those in the art.

In some embodiments, for example when second electron transfer moieties are not present, if m=0 then at least one of the G bonds is not an alkane bond.

In a preferred embodiment, the m of Structure 9 is zero. In a particularly preferred embodiment, m is zero and G is an alkene bond, as is depicted in Structure 10:

Structure 10

10

15

5

$$\left(\begin{array}{c} \\ \\ \\ \end{array}\right)_{n} \left(\begin{array}{c} \\ \\ \end{array}\right)_{m}$$

The alkene oligomer of structure 10, and others depicted herein, are generally depicted in the preferred trans configuration, although oligomers of cis or mixtures of trans and cis may also be used. As above, R groups may be added to alter the packing of the compositions on an electrode, the hydrophilicity or hydrophobicity of the oligomer, and the flexibility, i.e. the rotational, torsional or longitudinal flexibility of the oligomer. In is as defined above.

In a preferred embodiment, R is hydrogen, although R may be also alkyl groups and polyethylene glycols or derivatives.

In an alternative embodiment, the conductive oligomer may be a mixture of different types of oligomers, for example of structures 2 and 9.

25

The conductive oligomers are covalently attached to the nucleic acids. By "covalently attached" herein is meant that two moieties are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds.

The nucleic acid is covalently attached to the conductive oligomer, and the conductive oligomer is also covalently attached to the electrode. In general, the covalent attachments are done in such a manner as to minimize the amount of unconjugated sigma bonds an electron must travel from the electron donor to the electron acceptor. Thus, linkers are generally short, or contain conjugated bonds with few sigma bonds.

10

15

20

25

30

35

The covalent attachment of the nucleic acid and the conductive oligomer may be accomplished in several ways. In a preferred embodiment, the attachment is via attachment to the base of the nucleoside, via attachment to the backbone of the nucleic acid (either the ribose, the phosphate, or to an analogous group of a nucleic acid analog backbone), or via a transition metal ligand, as described below. The techniques outlined below are generally described for naturally occurring nucleic acids, although as will be appreciated by those in the art, similar techniques may be used with nucleic acid analogs.

In a preferred embodiment, the conductive oligomer is attached to the base of a nucleoside of the nucleic acid. This may be done in several ways, depending on the oligomer, as is described below. In one embodiment, the oligomer is attached to a terminal nucleoside, i.e. either the 3' or 5' nucleoside of the nucleic acid. Alternatively, the conductive oligomer is attached to an internal nucleoside.

The point of attachment to the base will vary with the base. While attachment at any position is possible, it is preferred to attach at positions not involved in hydrogen bonding to the complementary base. Thus, for example, generally attachment is to the 5 or 6 position of pyrimioines such as unidine, cytosine and thymine. For purines such as adenine and guanine, the linkage is preferably via the 8 position. Attachment to non-standard bases is preferably done at the comparable positions.

In one embodiment, the attachment is direct; that is, there are no intervening atoms between the conductive oligomer and the base. In this embodiment, for example, conductive oligomers with terminal acetylene bonds are attached directly to the base. Structure 11 is an example of this linkage, using a Structure 4 conductive oligomer and uridine as the base, although other bases and conductive oligomers can be used as will be appreciated by those in the art:

Structure 11

It should be noted that the pentose structures depicted herein may have hydrogen, hydroxy, phosphates or other groups such as amino groups attached. In addition, the pentose and nucleoside structures depicted herein are depicted non-conventionally, as mirror images of the normal rendering.

In addition, the pentose and nucleoside structures may also contain additional groups, such as protecting groups, at any position, for example as needed during synthesis.

In addition, the base may contain additional modifications as needed, i.e. the carbonyl or amine groups may be altered or protected, for example as is depicted in Figure 3 or 18.

In an alternative embodiment, the attachment is through an amide bond using a linker as needed, as is generally depicted in Structure 12 using uridine as the base and a Structure 4 oligomer:

Structure 12:

10

20

25

5

Preferred embodiments of Structure 12 include Z is a methylene or ethylene. The amide attachment can also be done using an amino group of the base, either a naturally occurring amino group such as in cytidine or adenidine, or from an amino-modified base as are known in the art.

In this embodiment, Z is a linker. Preferably, Z is a short linker of about 1 to about 5 atoms, that may or may not contain alkene bonds. Linkers are known in the art; for example, homo-or heterobifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). Preferred Z linkers include, but are not limited to, alkyl groups and alkyl groups containing heteroatom moieties, with short alkyl groups, esters, epoxy groups and ethylene glycol and derivatives being preferred, with propyl, acetylene, and C_2 alkene being especially preferred. Z may also be a sulfone group, forming sulfonamide linkages as discussed below.

In a preferred embodiment, the attachment of the nucleic acid and the conductive oligomer is done via attachment to the backbone of the nucleic acid. This may be done in a number of ways, including attachment to a ribose of the ribose-phosphate backbone, or to the phosphate of the backbone, or other groups of analogous backbones.

As a preliminary matter, it should be understood that the site of attachment in this embodiment may be to a 3' or 5' terminal nucleotide, or to an internal nucleotide, as is more fully described below.

35

In a preferred embodiment, the conductive oligomer is attached to the ribose of the ribose-phosphate backbone. This may be done in several ways. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose with amino groups, sulfur groups, silicone groups, phosphorus groups, or oxo groups can be made (Imazawa et al., J. Org. Chem., 44:2039 (1979); Hobbs et al., J. Org. Chem. 42(4):714 (1977); Verheyden et al., J. Orrg. Chem. 36(2):250 (1971); McGee et al., J. Org. Chem. 61:781-785 (1996); Mikhailopulo et al., Liebigs. Ann. Chem. 513-519 (1993); McGee et al., Nucleosides & Nucleotides 14(6):1329 (1995), all of which are incorporated by reference). These modified nucleosides are then used to add the conductive oligomers.

A preferred embodiment utilizes amino-modified nucleosides. These amino-modified riboses can then be used to form either amide or amine linkages to the conductive oligomers. In a preferred embodiment, the amino group is attached directly to the ribose, although as will be appreciated by those in the art, short linkers such as those described herein for "Z" may be present between the amino group and the ribose.

15

5

In a preferred embodiment, an amide linkage is used for attachment to the ribose. Preferably, if the conductive oligomer of Structures 2-4 is used, m is zero and thus the conductive oligomer terminates in the amide bond. In this embodiment, the nitrogen of the amino group of the amino-modified ribose is the "D" atom of the conductive oligomer. Thus, a preferred attachment of this embodiment is depicted in Structure 13 (using the Structure 4 conductive oligomer):

Structure 13

25

20

As will be appreciated by those in the art, Structure 13 has the terminal bond fixed as an amide bond.

In a preferred embodiment, a heteroatom linkage is used, i.e. oxo, amine, sulfur, etc. A preferred embodiment utilizes an amine linkage. Again, as outlined above for the amide linkages, for amine linkages, the nitrogen of the amino-modified ribose may be the "D" atom of the conductive oligomer when the Structure 4 conductive oligomer is used. Thus, for example, Structures 14 and 15 depict nucleosides with the Structures 4 and 10 conductive oligomers, respectively, using the nitrogen as the heteroatom, athough other heteroatoms can be used:

35

Structure 14

$$-(Y-B-D)$$
 (Y) (Y)

5

In Structure 14, preferably both m and t are not zero. A preferred Z here is a methylene group, or other aliphatic alkyl linkers. One, two or three carbons in this position are particularly useful for synthetic reasons; see Figure 16.

Structure 15

10

In Structure 15, Z is as defined above. Suitable linkers include methylene and ethylene.

15

In an alternative embodiment, the conductive oligomer is covalently attached to the nucleic acid via the phosphate of the ribose-phosphate backbone (or analog) of a nucleic acid. In this embodiment, the attachment is direct, utilizes a linker or via an amide bond. Structure 16 depicts a direct linkage, and Structure 17 depicts linkage via an amide bond (both utilize the Structure 4 conductive oligomer, although Structure 9 conductive oligomers are also possible). Structures 16 and 17 depict the conductive oligomer in the 3' position, although the 5' position is also possible. Furthermore, both Structures 16 and 17 depict naturally occurring phosphodiester bonds, although as those in the art will appreciate, non-standard analogs of phosphodiester bonds may also be used.

25

20

$$\frac{1}{\left(x-y-y-y\right)_{n}\left(x\right)_{n}\left(x\right)_{n}\left(x\right)_{n}}\left(x\right)_{n}\left(x\right)_{$$

Structure 16

30

In Structure 16, if the terminal Y is present (i.e. m=1), then preferably Z is not present (i.e. t=0). If the terminal Y is not present, then Z is preferably present.

35

Structure 17 depicts a preferred embodiment, wherein the terminal B-D bond is an amide bond, the terminal Y is not present, and Z is a linker, as defined herein.

In a preferred embodiment, the conductive oligomer is covalently attached to the nucleic acid via a transition metal ligand. In this embodiment, the conductive oligomer is covalently attached to a ligand which provides one or more of the coordination atoms for a transition metal. In one embodiment, the ligand to which the conductive oligomer is attached also has the nucleic acid attached, as is generally depicted below in Structure 18. Alternatively, the conductive oligomer is attached to one ligand, and the nucleic acid is attached to another ligand, as is generally depicted below in Structure 19. Thus, in the presence of the transition metal, the conductive oligomer is covalently attached to the nucleic acid. Both of these structures depict Structure 4 conductive oligomers, although other oligomers may be utilized. Structures 18 and 19 depict two representative structures:

Structure 18

$$\frac{1}{\sqrt{1+\frac{1}{2}}} \left(\frac{1}{2} \right) \left(\frac{1}{2$$

Structure 19

$$-\left(v-B-O\right)_{n}\left(v\right)_{m}\left(z\right)_{l}$$

In the structures depicted herein, M is a metal atom, with transition metals being preferred. Suitable transition metals for use in the invention include, but are not limited to, cadmium (Cd), copper (Cu), cobalt (Co), palladium (Pd), zinc (Zn), iron (Fe), ruthenium (Ru), rhodium (Rh), osmium (Os), rhenium (Re), platinium (Pt), scandium (Sc), titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), Molybdenium (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series

nickel (Ni), Molybdenum (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series of transition metals, the platinum metals (Ru, Rh, Pd, Os, Ir and Pt), along with Fe, Re, W, Mo and Tc, are preferred. Particularly preferred are ruthenium, rhenium, osmium, platinium, cobalt and iron.

L are the co-ligands, that provide the coordination atoms for the binding of the metal ion. As will be appreciated by those in the art, the number and nature of the co-ligands will depend on the

5

10

20

25

30

15

10

15

20

25

30

coordination number of the metal ion. Mono-, di- or polydentate co-ligands may be used at any position. Thus, for example, when the metal has a coordination number of six, the L from the terminus of the conductive oligomer, the L contributed from the nucleic acid, and r, add up to six. Thus, when the metal has a coordination number of six, r may range from zero (when all coordination atoms are provided by the other two ligands) to four, when all the co-ligands are monodentate. Thus generally, r will be from 0 to 8, depending on the coordination number of the metal ion and the choice of the other ligands.

In one embodiment, the metal ion has a coordination number of six and both the ligand attached to the conductive oligomer and the ligand attached to the nucleic acid are at least bidentate; that is, r is preferably zero, one (i.e. the remaining co-ligand is bidentate) or two (two monodentate co-ligands are used).

As will be appreciated in the art, the co-ligands can be the same or different. Suitable ligands fall into two categories: ligands which use nitrogen, oxygen, sulfur, carbon or phosphorus atoms (depending on the metal ion) as the coordination atoms (generally referred to in the literature as sigma (σ) donors) and organometallic ligands such as metallocene ligands (generally referred to in the literature as pi (π) donors, and depicted herein as L_m). Suitable nitrogen donating ligands are well known in the art and include, but are not limited to, NH₂; NHR; NRR'; pyridine; pyrazine; isonicotinamide; imidazole; bipyridine and substituted derivatives of bipyridine; terpyridine and substituted derivatives; phenanthrolines, particularly 1,10-phenanthroline (abbreviated phen) and substituted derivatives of phenanthrolines such as 4,7-dimethylphenanthroline and dipyridol[3,2-a:2',3'-c]phenazine (abbreviated dppz); dipyridophenazine; 1,4,5,8,9,12-hexaazatriphenylene (abbreviated hat); 9,10phenanthrenequinone diimine (abbreviated phi); 1,4,5,8-tetraazaphenanthrene (abbreviated tap); 1,4,8,11-tetra-azacyclotetradecane (abbreviated cyclam) and isocyanide. Substituted derivatives, including fused derivatives, may also be used. In some embodiments, porphyrins and substituted derivatives of the porphyrin family may be used. See for example, Comprehensive Coordination Chemistry, Ed. Wilkinson et al., Pergammon Press, 1987, Chapters 13.2 (pp73-98), 21.1 (pp. 813-898) and 21.3 (pp 915-957), all of which are hereby expressly incorporated by reference.

Suitable sigma donating ligands using carbon, oxygen, sulfur and phosphorus are known in the art. For example, suitable sigma carbon donors are found in Cotton and Wilkenson, Advanced Organic Chemistry. 5th Edition, John Wiley & Sons, 1988, hereby incorporated by reference; see page 38, for example. Similarly, suitable oxygen ligands include crown ethers, water and others known in the art. Phosphines and substituted phosphines are also suitable; see page 38 of Cotton and Wilkenson.

10

15

20

25

30

35

The oxygen, sulfur, phosphorus and nitrogen-donating ligands are attached in such a manner as to allow the heteroatoms to serve as coordination atoms.

In a preferred embodiment, organometallic ligands are used. In addition to purely organic compounds for use as redox moieties, and various transition metal coordination complexes with 5-bonded organic ligand with donor atoms as heterocyclic or exocyclic substituents, there is available a wide variety of transition metal organometallic compounds with π-bonded organic ligands (see Advanced Inorganic Chemistry, 5th Ed., Cotton & Wilkinson, John Wiley & Sons, 1988, chapter 26; Organometallics, A Concise Introduction, Elschenbroich et al., 2nd Ed., 1992, VCH; and Comprehensive Organometallic Chemistry II, A Review of the Literature 1982-1994, Abel et al. Ed., Vol. 7, chapters 7, 8, 10 & 11. Pergamon Press, hereby expressly incorporated by reference). Such organometallic ligands include cyclic aromatic compounds such as the cyclopentadienide ion [C₅H₅(-1)] and various ring substituted and ring fused derivatives, such as the indenylide (-1) ion, that yield a class of bis(cyclopentadieyl)metal compounds, (i.e. the metallocenes); see for example Robins et al., J. Am. Chem. Soc. 104:1882-1893 (1982); and Gassman et al., J. Am. Chem. Soc. 108:4228-4229 (1986), incorporated by reference. Of these, ferrocene [(C₅H₅)₂Fe] and its derivatives are prototypical examples which have been used in a wide variety of chemical (Connelly et al., Chem. Rev. 96:877-910 (1996), incorporated by reference) and electrochemical (Geiger et al., Advances in Organometallic Chemistry 23:1-93; and Geiger et al., Advances in Organometallic Chemistry 24:87, incorporated by reference) electron transfer or "redox" reactions. Metallocene derivatives of a variety of the first, second and third row transition metals are potential candidates as redox moieties that are covalently attached to either the ribose ring or the nucleoside base of nucleic acid. Other potentially suitable organometallic ligands include cyclic arenes such as benzene, to yield bis(arene)metal compounds and their ring substituted and ring fused derivatives, of which bis(benzene)chromium is a prototypical example. Other acyclic π-bonded ligands such as the allyl(-1) ion, or butadiene yield potentially suitable organometallic compounds, and all such ligands, in conjuction with other π -bonded and δ bonded ligands constitute the general class of organometallic compounds in which there is a metal to carbon bond. Electrochemical studies of various dimers and oligomers of such compounds with bridging organic ligands, and additional non-bridging ligands, as well as with and without metal-metal bonds are potential candidate redox moieties in nucleic acid analysis.

When one or more of the co-ligands is an organometallic ligand, the ligand is generally attached via one of the carbon atoms of the organometallic ligand, although attachment may be via other atoms for heterocyclic ligands. Preferred organometallic ligands include metallocene ligands, including substituted derivatives and the metalloceneophanes (see page 1174 of Cotton and Wilkenson, supra). For example, derivatives of metallocene ligands such as methylcyclopentadienyl, with multiple methyl

groups being preferred, such as pentamethylcyclopentadienyl, can be used to increase the stability of the metallocene. In a preferred embodiment, only one of the two metallocene ligands of a metallocene are derivatized.

As described herein, any combination of ligands may be used. Preferred combinations include: a) all ligands are nitrogen donating ligands; b) all ligands are organometallic ligands; and c) the ligand at the terminus of the conductive oligomer is a metallocene ligand and the ligand provided by the nucleic acid is a nitrogen donating ligand, with the other ligands, if needed, are either nitrogen donating ligands or metallocene ligands, or a mixture. These combinations are depicted in representative structures using the conductive oligomer of Structure 4 are depicted in Structures 20 (using phenanthroline and amino as representative ligands), 21 (using ferrocene as the metal-ligand combination) and 22 (using cyclopentadienyl and amino as representative ligands).

Structure 20

15

10

5

$$-(v-a-b)_n(v)_m(z)_t$$

Structure 21

20

25

30

35

In a preferred embodiment, the ligands used in the invention show altered fluoroscent properties depending on the redox state of the chelated metal ion. As described below, this thus serves as an additional mode of detection of electron transfer through nucleic acid.

In a preferred embodiment, as is described more fully below, the ligand attached to the nucleic acid is an amino group attached to the 2' or 3' position of a ribose of the ribose-phosphate backbone. This

10

15

20

25

ligand may contain a multiplicity of amino groups so as to form a polydentate ligand which binds the metal ion. Other preferred ligands include cyclopentadiene and phenanthroline.

As described herein, the compositions described herein of nucleosides covalently attached to conductive oligomers may be incorporated into a longer nucleic acid at any number of positions, including either the 5' or 3' terminus of the nucleic acid or any internal position. As is outlined below, this is generally done by adding a nucleotide with a covalently attached conductive oligomer to an oligonucleotide synthetic reaction at any position. After synthesis is complete, the nucleic acid with the covalently attached conductive oligomer is attached to an electrode. Thus, any number of additional nucleotides, modified or not, may be included at any position. Alternatively, the compositions are made via post-nucleic acid synthesis modifications.

The total length of the nucleic acid will depend on its use. Generally, the nucleic acid compositions of the invention are useful as oligonucleotide probes. As is appreciated by those in the art, the length of the probe will vary with the length of the target sequence and the hybridization and wash conditions. Generally, oligonucleotide probes range from about 8 to about 50 nucleotides, with from about 10 to about 30 being preferred and from about 12 to about 25 being especially preferred. In some cases, very long probes may be used, e.g. 50 to 200-300 nucleotides in length.

Also of consideration is the distance between the nucleoside containing the electrode, i.e. a first electron transfer moiety, and the nucleoside containing a second electron transfer moiety. Electron transfer proceeds between the two electron transfer moieties. Since the rate of electron transfer is distance dependent, the distance between the two electron transfer moieties preferably ranges from about 1 to about 30 basepairs, with from about 1 to about 20 basepairs being preferred and from about 2 to about 10 basepairs being particularly preferred and from about 2 to 6 being especially preferred. However, probe specificity can be increased by adding oligonucleotides on either side of the electron transfer moieties, thus increasing probe specificity without increasing the distance an electron must travel.

Thus, in the structures depicted herein, nucleosides may be replaced with nucleic acids.

In a preferred embodiment, the conductive oligomers with covalently attached nucleosides or nucleic acids as depicted herein are covalently attached to an electrode. Thus, one end or terminus of the conductive oligomer is attached to the nucleoside or nucleic acid, and the other is attached to an electrode. In some embodiments it may be desirable to have the conductive oligomer attached at a position other than a terminus, or even to have a branched conductive oligomer that is attached to an

10

15

20

25

electrode at one terminus and to two or more nucleosides at other termini, although this is not preferred. Similarly, the conductive oligomer may be attached at two sites to the electrode.

By "electrode" herein is meant a composition, which, when connected to an electronic device, is able to sense a current or charge and convert it to a signal. Thus, an electrode is an electron transfer moiety as described herein. Preferred electodes are known in the art and include, but are not limited to, certain metals and their oxides, including gold; platinum; palladium; silicon; aluminum; metal oxide electrodes including platinum oxide, titanium oxide, tin oxide, indium tin oxide, palladium oxide, silicon oxide, aluminum oxide, molybdenum oxide (Mo₂O₆), tungsten oxide (WO₃) and ruthenium oxides; and carbon (including glassy carbon electrodes, graphite and carbon paste). Preferred electrodes include gold, silicon, carbon and metal oxide electrodes.

The electrodes described herein are depicted as a flat surface, which is only one of the possible conformations of the electrode and is for schematic purposes only. The conformation of the electrode will vary with the detection method used. For example, flat planar electrodes may be preferred for optical detection methods, or when arrays of nucleic acids are made, thus requiring addressable locations for both synthesis and detection. Alternatively, for single probe analysis, the electrode may be in the form of a tube, with the conductive oligomers and nucleic acids bound to the inner surface. This allows a maximum of surface area containing the nucleic acids to be exposed to a small volume of sample.

The covalent attachment of the conductive oligomer containing the nucleoside may be accomplished in a variety of ways, depending on the electrode and the conductive oligomer used. Generally, some type of linker is used, as depicted below as "A" in Structure 23, where X is the conductive oligomer, and the hatched surface is the electrode:

Structure 23

A X — nucleoside

30

35

In this embodiment, A is a linker or atom. The choice of "A" will depend in part on the characteristics of the electrode. Thus, for example, A may be a sulfur moiety when a gold electrode is used. Alternatively, when metal oxide electrodes are used, A may be a silicon (silane) moiety attached to the oxygen of the oxide (see for example Chen et al., Langmuir 10:3332-3337 (1994); Lenhard et al., J. Electroanal. Chem. 78:195-201 (1977), both of which are expressly incorporated by reference). When

carbon based electrodes are used, A may be an amino moiety (preferably a primary amine; see for example Deinhammer et al., Langmuir 10:1306-1313 (1994)). Thus, preferred A moieties include, but are not limited to, silane moieties, sulfur moieties (including alkyl sulfur moieties), and amino moieties. In a preferred embodiment, epoxide type linkages with redox polymers such as are known in the art are not used.

Although depicted herein as a single moiety, the conductive oligomer may be attached to the electrode with more than one "A" moiety; the "A" moieties may be the same or different. Thus, for example, when the electrode is a gold electrode, and "A" is a sulfur atom or moiety, such as generally depicted below in Structure 27, multiple sulfur atoms may be used to attach the conductive oligomer to the electrode, such as is generally depicted below in Structures 24, 25 and 26. As will be appreciated by those in the art, other such structures can be made. In Structures 24, 25 and 26, the A moiety is just a sulfur atom, but substituted sulfur moieties may also be used.

Structure 24

15

5

10

Structure 25

20

Structure 26

25

30

35

It should also be noted that similar to Structure 26, it may be possible to have a a conductive oligomer terminating in a single carbon atom with three sulfur moities attached to the electrode.

In a preferred embodiment, the electrode is a gold electrode, and attachment is via a sulfur linkage as is well known in the art, i.e. the A moiety is a sulfur atom or moiety. Although the exact characteristics of the gold-sulfur attachment are not known, this linkage is considered covalent for the purposes of this invention. A representative structure is depicted in Structure 27 Structure 27 depicts the "A"

WO 98/20162

linker as comprising just a sulfur atom, although additional atoms may be present (i.e. linkers from the sulfur to the conductive oligomer or substitution groups).

-32-

Structure 27

5

In a preferred embodiment, the electrode is a carbon electrode, i.e. a glassy carbon electrode, and attachment is via a nitrogen of an amine group. A representative structure is depicted in Structure 28. Again, additional atoms may be present, i.e. Z type linkers.

Structure 28

15

10

NHL
$$\left(Y - B - O \right)_{n} \left(Y \right)_{m} \left(Z \right)_{t}$$
 nucleic acid

Structure 29

20

25

30

35

$$O - S_1 - \left(Y - B - O\right)_{n} \left(Y - \frac{Z}{m}\right)_{t}$$
 reucleic acid

In Structure 29, the oxygen atom is from the oxide of the metal oxide electrode. The Si atom may also contain other atoms, i.e. be a silicon moiety containing substitution groups.

Thus, in a preferred embodiment, electrodes are made that comprise conductive oligomers attached to nucleic acids for the purposes of hybridization assays as is more fully described herein. As will be appreciated by those in the art, electrodes can be made that have a single species of nucleic acid, i.e. a single nucleic acid sequence, or multiple nucleic acid species.

In addition, as outlined herein, the use of a solid support such as an electrode enables the use of these gene probes in an array form. The use of oligonucleotide arrays are well known in the art. In addition, techniques are known for "addressing" locations within an electrode and for the surface modification of electrodes. Thus, in a preferred embodiment, arrays of different nucleic acids are laid down on the electrode, each of which are covalently attached to the electrode via a conductive linker.

10

15

20

25

In this embodiment, the number of different probe species of oligonucleotides may vary widely, from one to thousands, with from about 4 to about 100,000 being preferred, and from about 10 to about 10,000 being particularly preferred.

In a preferred embodiment, the electrode further comprises a passivation agent, preferably in the form of a monolayer on the electrode surface. As outlined above, the efficiency of oligonucleotide hybridization may increase when the oligonucleotide is at a distance from the electrode. A passivation agent layer facilitates the maintenance of the nucleic acid away from the electrode surface. In addition, a passivation agent serves to keep charge carriers away from the surface of the electrode. Thus, this layer helps to prevent electrical contact between the electrodes and the electron transfer moieties, or between the electrode and charged species within the solvent. Such contact can result in a direct "short circuit" or an indirect short circuit via charged species which may be present in the sample. Accordingly, the monolayer of passivation agents is preferably tightly packed in a uniform layer on the electrode surface, such that a minimum of "holes" exist. Alternatively, the passivation agent may not be in the form of a monolayer, but may be present to help the packing of the conductive oligomers or other characteristics.

The passivation agents thus serve as a physical barrier to block solvent accesibility to the electrode. As such, the passivation agents themselves may in fact be either (1) conducting or (2) nonconducting, i.e. insulating, molecules. Thus, in one embodiment, the passivation agents are conductive oligomers, as described herein, with or without a terminal group to block or decrease the transfer of charge to the electrode. Other passivation agents which may be conductive include oligomers of $-(CF_2)_{n^{-1}}$ and $-(CFR)_{n^{-1}}$. In a preferred embodiment, the passivation agents are insulator moieties.

An "insulator" is a substantially nonconducting oligomer, preferably linear. By "substantially nonconducting" herein is meant that the rate of electron transfer through the insulator is slower than the rate of electron transfer through the stacked ri-orbitals of double stranded nucleic acid. Stated differently, the electrical resistance of the insulator is higher than the electrical resistance of the nucleic acid. In a preferred embodiment, the rate of electron transfer through the insulator is slower than or comparable to the rate through single stranded nucleic acid. Similarly, the rate of electron transfer through the insulator is preferrably slower than the rate through the conductive oligomers described herein. It should be noted however, as outlined in the Examples, that even oligomers generally considered to be insulators, such as -(CH₂)₁₆ molecules, still may transfer electrons, albeit at a slow rate.

35

In a preferred embodiment, the insulators have a conductivity, S, of about $10^{-7} \,\Omega^{-1} \text{cm}^{-1}$ or lower, with less than about $10^{-8} \,\Omega^{-1} \text{cm}^{-1}$ being preferred. See generally Gardner et al., supra.

Generally, insulators are alkyl or heteroalkyl oligomers or moieties with sigma bonds, although any particular insulator molecule may contain aromatic groups or one or more conjugated bonds. By "heteroalkyl" herein is meant an alkyl group that has at least one heteroatom, i.e. nitrogen, oxygen, sulfur, phosphorus, silicon or boron included in the chain. Alternatively, the insulator may be quite similar to a conductive oligomer with the addition of one or more heteroatoms or bonds that serve to inhibit or slow, preferably substantially, electron transfer.

10

15

20

25

5

The passivation agents, including insulators, may be substituted with R groups as defined herein to alter the packing of the moieties or conductive oligomers on an electrode, the hydrophilicity or hydrophobicity of the insulator, and the flexibility, i.e. the rotational, torsional or longitudinal flexibility of the insulator. For example, branched alkyl groups may be used. In addition, the terminus of the passivation agent, including insulators, may contain an additional group to influence the exposed surface of the monolayer. For example, there may be negatively charged groups on the terminus to form a negatively charged surface such that when the nucleic acid is DNA or RNA the nucleic acid is repelled or prevented from lying down on the surface, to facilitate hybridization. Preferred passivation agent terminal groups include -NH₂, -OH, -COOH, -CH₃, and (poly)alkyloxides such as (poly)ethylene glycol, with -OCH₂CH₂OH, -(OCH₂CH₂O)₂H and -(OCH₂CH₂O)₃H being preferred and the latter being particularly preferred.

The length of the passivation agent will vary as needed. As outlined above, it appears that hybridization is more efficient at a distance from the surface. Thus, the length of the passivation agents is similar to the length of the conductive oligomers, as outlined above. In addition, the conductive oligomers may be basically the same length as the passivation agents or longer than them, resulting in the nucleic acids being more accessible to the solvent for hybridization.

30

The monolayer may comprise a single type of passivation agent, including insulators, or different types.

Suitable insulators are known in the art, and include, but are not limited to, $-(CH_2)_{n^-}$, $-(CRH)_{n^-}$, and $-(CR_2)_{n^-}$, ethylene glycol or derivatives using other heteroatoms in place of oxygen, i.e. nitrogen or sulfur (sulfur derivatives are not preferred when the electrode is gold).

10

15

20

25

30

35

The passivation agents are generally attached to the electrode in the same manner as the conductive oligomer, and may use the same "A" linker as defined above.

It has been found that the present compositions result in excellent hybridization kinetics of target sequence hybridizing to probes attached to a surface. Thus, the compositions and methods of the present invention may also be used in nucleic acid detection systems that do not rely on electron transfer for detection.

Accordingly, in a preferred embodiment, the compositions of the present invention find use in standard nucleic acid assays, such as general array-type technologies, i.e. the electrode may serve just as a solid support, with detection proceeding using techniques well known in the art, such as fluoroscence or radioisotope labelling. In this embodiment, the compositions may comprise a conductive oligomer covalently attached to a nucleoside or nucleic acid. It will be recognized by those in the art that the conductive oligomers in this embodiment may not be functioning as conductive oligomers but rather as linkers that can be used to keep the nucleic acids off the surface. The conductive oligomer, or linker, in this case may have the structure depicted in Structures 2, 3, 4, 9 or 10. However, when the linker has the structure depicted in Structure 9, preferably at least one of the G bonds is not alkane, particularly when m=0.

In a preferred embodiment, the composition comprises (a) a solid support comprising a monolayer of passivation agent; (b) a nucleic acid comprising at least one nucleoside, wherein the nucleic acid is covalently attached to the solid support with a linker. The solid support is the electrode, which is not necessarily functioning as an electron transfer moiety in this embodiment. The monolayer of passivation agent is shown herein to result in excellent hybridization kinetics and can therefore be quite useful in both electron-transfer based and traditional nucleic acid detection schemes. The linkers are preferably the conductive oligomers of the invention, although as outlined above, they may not be functioning as conductive moieties. In this embodiment, the conductive oligomer, or linker, in this case, may have the structure depicted in Structures 2, 3, 4, 9 or 10. However, when the linker has the structure depicted in Structure 3, preferably at least one of the G bonds is not alkane, particularly when m=0.

In this embodiment, it is possible to have each nucleic acid be the same, as an "anchor sequence", such that a second sequence can be added which contains the probe sequence and a sequence complementary to the anchor sequence. In this way, standard arrays of using either the same or different anchor sequences can be made, which then can be used to generate custom arrays using novel probe sequences linked to complementary anchor regions.

Thus, in this embodiment, compositions are provided comprising a conductive oligomer covalently attached to an electrode and to a first single stranded anchor sequence. A second single stranded nucleic acid is provided, which contains a probe region and a region substantially complementary to the anchor sequence, such that a first hybridization complex is formed between the two complementary anchor regions, leaving the probe region as a single stranded region. A target sequence which is substantially complementary to the probe region is then added to form a second hybridization complex. The second hybridization complex is then detected, for example by labelling the target nucleic acid as is well known in the art.

As outlined herein, it is also possible to have compositions comprising electrodes with conductive oligomers attached to probe nucleic acids, without second electron transfer moieties, and soluble second probe sequences with second electron transfer moieties. Upon binding of the target sequence, which contains a first target domain for the first probe sequence and a second target domain for the second probe sequence, which preferably are adjacent, electron transfer may occur.

15

10

5

Alternatively, it may be the target sequence which contains the second electron transfer moiety Similar to methods which rely on amplification and labelling of target sequences, the target nucleic acid may be labelled with a second electron transfer moiety which then can be used to effect electron transfer upon formation of the hybridization complex.

20

25

In an alternate embodiment, a hybridization indicator may serve as either the sole second electron transfer moiety or as an additional second electron transfer moiety, as is generally described below.

In a preferred embodiment, the compositions of the present invention comprise a conductive oligomer, covalently attached to both an electrode, which serves as a first electron transfer moiety, and a nucleic acid, which has at least a second covalently attached electron transfer moiety. As noted herein, the conductive oligomer and the second electron transfer moiety may be attached at any position of the nucleic acid.

30

35

In one embodiment, a nucleic acid is modified with more than two electron transfer moieties. For example, to increase the signal obtained from the probe, or alter the required detector sensitivity, a plurality of electron transfer moieties may be used. See PCT publication WO 95/15971. For example, the conductive oligomer may be attached to an internal nucleoside, with second electron transfer moieties (ETM) attached both 5' and 3' to the nucleoside containing the conductive oligomer, as is generally depicted in Structure 29A. In one embodiment, the two additional electron transfer moieties are the same, and are placed the same distance away from the conductive oligomer, to result in a

uniform signal. Alternatively, the additional electron transfer moieties may be different and/or placed at different distances from the conductive oligomer.

Structure 29A

5

10

15

The terms "electron donor moiety", "electron acceptor moiety", and "electron transfer moieties" or grammatical equivalents herein refers to molecules capable of electron transfer under certain conditions. It is to be understood that electron donor and acceptor capabilities are relative; that is, a molecule which can lose an electron under certain experimental conditions will be able to accept an electron under different experimental conditions. It is to be understood that the number of possible electron donor moieties and electron acceptor moieties is very large, and that one skilled in the art of electron transfer compounds will be able to utilize a number of compounds in the present invention. Preferred electron transfer moieties include, but are not limited to, transition metal complexes, organic electron transfer moieties, and electrodes.

20

In a preferred embodiment, the electron transfer moieties are transition metal complexes. Transition metals are those whose atoms have a partial or complete d shell of electrons. Suitable transition metals for use in the invention are listed above.

25

The transition metals are complexed with a variety of ligands, L, defined above, to form suitable transition metal complexes, as is well known in the art.

30

In addition to transition metal complexes, other organic electron donors and acceptors may be covalently attached to the nucleic acid for use in the invention. These organic molecules include, but are not limited to, riboflavin, xanthene dyes, azine dyes, acridine orange, *N*,*N*-dimethyl-2,7-diazapyrenium dichloride (DAP²*), methylviologen, ethidium bromide, quinones such as N,N'-dimethylanthra(2,1,9-def.6,5,10-d'e'f')diisoquinoline dichloride (ADIQ²*); porphyrins ([meso-tetrakis(N-methyl-x-pyridinium)porphyrin tetrachloride], varlamine blue B hydrochloride, Bindschedler's green; 2,6-dichloroindophenol, 2,6-dibromophenolindophenol; Brilliant crest blue (3-amino-9-dimethyl-amino-10-methylphenoxyazine chloride), methylene blue, Nile blue A (aminoaphthodiethylaminophenoxazine

10

15

20

25

30

sulfate), indigo-5,5',7,7'-tetrasulfonic acid, indigo-5,5',7-trisulfonic acid; phenosafranine, indigo-5-monosulfonic acid; safranine T; bis(dimethylglyoximato)-iron(II) chloride; induline scarlet, neutral red, anthracene, coronene, pyrene, 9-phenylanthracene, rubrene, binaphthyl, DPA, phenothiazene, fluoranthene, phenanthrene, chrysene, 1,8-diphenyl-1,3,5,7-octatetracene, naphthalene, acenaphthalene, perylene, TMPD and analogs and substitiuted derivatives of these compounds.

In one embodiment, the electron donors and acceptors are redox proteins as are known in the art. However, redox proteins in many embodiments are not preferred.

The choice of the specific electron transfer moieties will be influenced by the type of electron transfer detection used, as is generally outlined below.

In a preferred embodiment, these electron transfer moieties are covalently attached to the nucleic acid in a variety of positions. In a preferred embodiment, the attachment is via attachment to the base of the nucleoside, or via attachment to the backbone of the nucleic acid, including either to a ribose of the ribose-phosphate backbone or to a phosphate moiety. In the preferred embodiments, the compositions of the invention are designed such that the electron transfer moieties are as close to the "m-way" as possible without significantly disturbing the secondary and tertiary structure of the double helical nucleic acid, particularly the Watson-Crick basepairing. Alternatively, the attachment can be via a conductive oligomer, which is used as outlined above with a nucleoside and an electrode; that is, an electron transfer moiety may be covalently attached to a conductive oligomer at one end and to a nucleoside at the other, thus forming a general structure depicted in Structure 30:

Structure 30

A X mucleoside (mucleoside

In Structure 30, ETM is an electron transfer moiety, X is a conductive oligomer, and q is an integer from zero to about 25, with preferred q being from about 2 to about 10. Additionally, linker moieties, for example as are generally described herein as "Z", may also be present between the nucleoside and the conductive oligomer, and/or between the conductive oligomer and the electron transfer moiety. The depicted nucleosides may be either terminal or internal nucleosides, and are usually separated by a number of nucleosides.

In a preferred embodiment, the second electron transfer moiety is attached to the base of a nucleoside, as is generally outlined above for attachment of the conductive oligomer. This is preferably done to the base of an internal nucleoside. Surprisingly and unexpectedly, this attachment does not perturb the Watson-Crick basepairing of the base to which the electron transfer moiety is attached, as long as the moiety is not too large. In fact, it appears that attachment at this site actually results in less perturbation than attachment at the ribose of the ribose-phosphate backbone, as measured by nucleic acid melting curves.

Thus, when attachment to an internal base is done, the size of the second electron transfer moiety should be such that the structure of double stranded nucleic acid containing the base-attached electron transfer moiety is not significantly disrupted, and will not disrupt the annealing of single stranded nucleic acids. Preferrably, then, ligands and full second electron transfer moieties are generally smaller than the size of the major groove of double stranded nucleic acid.

Alternatively, the second electron transfer moiety can be attached to the base of a terminal nucleoside. Thus, when the target sequence to be detected is ninucleosides long, a probe can be made which has the second electron transfer moiety attached at the nibase. Alternatively, the probe may contain an extra terminal nucleoside at an end of the nucleic acid (n + 1 or n + 2), which are used to covalently attach the electron transfer moieties but which do not participate in basepair hybridization. Additionally, it is preferred that upon probe hybridization, the terminal nucleoside containing the electron transfer moiety covalently attached at the base be directly adjacent to Watson-Crick basepaired nucleosides; that is, the electron transfer moiety should be as close as possible to the stacked π-orbitals of the bases such that an electron travels through a minimum of σ bonds to reach the "π-way", or alternatively can otherwise electronically contact the π-way.

25

30

35

5

10

15

20

The covalent attachment to the base will depend in part on the second electron transfer moiety chosen, but in general is similar to the attachment of conductive oligomers to bases, as outlined above. In a preferred embodiment, the second electron transfer moiety is a transition metal complex, and thus attachment of a suitable metal ligand to the base leads to the covalent attachment of the electron transfer moiety. Alternatively, similar types of linkages may be used for the attachment of organic electron transfer moieties, as will be appreciated by those in the art.

In one embodiment, the C4 attached amino group of cytosine, the C6 attached amino group of adenine, or the C2 attached amino group of guanine may be used as a transition metal ligand, although in this embodiment attachment at a terminal base is preferred since attachment at these positions will perturb Watson-Crick basepairing.

Ligands containing aromatic groups can be attached via acetylene linkages as is known in the art (see Comprehensive Organic Synthesis, Trost et al., Ed., Pergamon Press, Chapter 2.4: Coupling Reactions Between sp² and sp Carbon Centers, Sonogashira, pp521-549, and pp950-953, hereby incorporated by reference). Structure 31 depicts a representative structure in the presence of the metal ion and any other necessary ligands; Structure 31 depicts uridine, although as for all the structures herein, any other base may also be used.

Structure 31

N N

L_a is a ligand, which may include nitrogen, oxygen, sulfur or phosphorus donating ligands or organometallic ligands such as metallocene ligands. Suitable L_a ligands include, but not limited to, phenanthroline, imidazole, bpy and terpy. L, and M are as defined above. Again, it will be appreciated by those in the art, a conductive oligomer may be included between the nucleoside and the electron transfer moiety.

Similarly, as for the conductive oligomers, the linkage may be done using a linker, which may utilize an amide linkage (see generally Telser et al., J. Am. Chem. Soc. 111:7221-7226 (1989); Telser et al., J. Am. Chem. Soc. 111:7226-7232 (1989), both of which are expressly incorporated by reference). These structures are generally depicted below in Structure 32, which again uses uridine as the base, although as above, the other bases may also be used:

Structure 32

In this embodiment, L is a ligand as defined above, with L, and M as defined above as well. Preferably, L is amino, phen, byp and terpy.

10

5

20

30

35

In a preferred embodiment, the second electron transfer moiety attached to a nucleoside is a metallocene; i.e. the L and L, of Structure 32 are both metallocene ligands, L_m , as described above. Structure 33 depicts a preferred embodiment wherein the metallocene is ferrocene, and the base is uridine, although other bases may be used:

Structure 33

10

5

Preliminary data suggest that Structure 33 may cyclize, with the second acetylene carbon atom attacking the carbonyl oxygen, forming a furan-like structure.

Thus, in a preferred embodiment, the invention provides metallocenes covalently attached to

15

Preferred metallocenes include ferrocene, cobaltocene and osmiumocene.

nucleosides. In a preferred embodiment, the metallocene is attached to the base of a nucleoside. In alternate preferred embodiment, the metallocene is attached to the ribose of a nucleoside. Alternatively, the metallocene may be attached to the phosphate of the backbone, although this is generally not preferred. If attachment is to the phosphate, generally there will be no more than about 2-4 atoms between the phosphate atom and a carbon of a ring of the metallocene. In a preferred

embodiment, the metallocene is ferrocene or substituted ferrocene.

25

30

35

20

In a preferred embodiment, the second electron transfer moiety is attached to a ribose at any position of the ribose-phosphate backbone of the nucleic acid, i.e. either the 5' or 3' terminus or any internal nucleoside. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose can be made, with nitrogen, oxygen, sulfur and phosphorus-containing modifications possible. Amino-modified ribose is preferred. See generally PCT publication WO 95/15971, incorporated herein by reference. These modification groups may be used as a transition metal ligand, or as a chemically functional moiety for attachment of other transition metal ligands and organometallic ligands, or organic electron donor moieties as will be appreciated by those in the art. In this embodiment, a linker such as depicted herein for "Z" may be used as well, or a conductive oligomer between the ribose and the electron transfer moiety. Preferred embodiments utilize attachment at the 2' or 3' position of the ribose, with the 2' position being preferred. Thus for example, the conductive oligomers depicted in

-42-

PCT/US97/20014 -

Structure 13, 14 and 15 may be replaced by electron transfer moieties; alternatively, as is depicted in Structure 30, the electron transfer moieties may be added to the free terminus of the conductive oligomer.

In a preferred embodiment, a metallocene serves as the second electron transfer moiety, and is attached via an amide bond as depicted below in Structure 34. The examples outline the synthesis of a preferred compound when the metallocene is ferrocene.

10

15

20

Amine linkages, or linkages via other heteroatoms, are also possible.

In a preferred embodiment, the second electron transfer moiety is attached to a phosphate at any position of the ribose-phosphate backbone of the nucleic acid. This may be done in a variety of ways. In one embodiment, phosphodiester bond analogs such as phosphoramide or phosphoramidite linkages may be incorporated into a nucleic acid as a transition metal ligand (see PCT publication WO 95/15971, incorporated by reference). Alternatively, the conductive oligomers depicted in Structures 16 and 17 may be replaced by electron transfer moieties; alternatively, the electron transfer moieties may be added to the free terminus of the conductive oligomer.

25

30

Preferred electron transfer moieties for covalent attachment to a single stranded nucleic acid include, but are not limited to, transition metal complexes, including metallocenes and substituted metallocenes such as metalloceneophanes, and complexes of Ru, Os, Re and Pt. Particularly preferred are ferrocene and its derivatives (particularly pentamethylferrocene and ferroceneophane) and complexes of transition metals including Ru, Os, Re and Pt containing one or more amine or polyamine, imidazole, phenathroline, pyridine, bipyridine and or terpyridine and their derivatives. For Pt, additional preferred ligands include the diimine dithiolate complexes such as quinoxaline-2,3-dithiolate complexes.

As described herein, the invention provides compositions containing electrodes as a first electron transfer moiety linked via a conductive oligomer to a nucleic acid which has at least a second electron

transfer moiety covalently attached. Any combination of positions of electron transfer moiety attachment can be made; i.e. an electrode at the 5' terminus, a second electron transfer moiety at an internal position; electrode at the 5' terminus, second moiety at the 3' end; second moiety at the 5' terminus, electrode at an internal position; both electrode and second moiety at internal positions; electrode at an internal position, second moiety at the 3' terminus, etc. A preferred embodiment utilizes both the electrode and the second electron transfer moiety attached to internal nucleosides.

The compositions of the invention may additionally contain one or more labels at any position. By "label" herein is meant an element (e.g. an isotope) or chemical compound that is attached to enable the detection of the compound. Preferred labels are radioactive isotopic labels, and colored or fluorescent dyes. The labels may be incorporated into the compound at any position. In addition, the compositions of the invention may also contain other moieties such as cross-linking agents to facilitate cross-linking of the target-probe complex. See for example, Lukhtanov et al., Nucl. Acids. Res. 24(4):683 (1996) and Tabone et al., Biochem. 33:375 (1994), both of which are expressly incorporated by reference.

The compositions of the invention are generally synthesized as outlined below, generally utilizing techniques well known in the art. As will be appreciated by those in the art, many of the techniques outlined below are directed to nucleic acids containing a ribose-phosphate backbone. However, as outlined above, many alternate nucleic acid analogs may be utilized, some of which may not contain either ribose or phosphate in the backbone. In these embodiments, for attachment at positions other than the base, attachment is done as will be appreciated by those in the art, depending on the backbone. Thus, for example, attachment can be made at the carbon atoms of the PNA backbone, as is described below, or at either terminus of the PNA.

25

30

35

5

10

15

20

The compositions may be made in several ways. A preferred method first synthesizes a conductive oligomer attached to the nucleoside, with addition of additional nucleosides followed by attachment to the electrode. A second electron transfer moiety, if present, may be added prior to attachment to the electrode or after. Alternatively, the whole nucleic acid may be made and then the completed conductive oligomer added, followed by attachment to the electrode. Alternatively, the conductive oligomer and monolayer (if present) are attached to the electrode first, followed by attachment of the nucleic acid. The latter two methods may be preferred when conductive oligomers are used which are not stable in the solvents and under the conditions used in traditional nucleic acid synthesis.

In a preferred embodiment, the compositions of the invention are made by first forming the conductive oligomer covalently attached to the nucleoside, followed by the addition of additional nucleosides to

form a nucleic acid, including, if present, a nucleoside containing a second electron transfer moiety, with the last step comprising the addition of the conductive oligomer to the electrode.

The attachment of the conductive oligomer to the nucleoside may be done in several ways. In a preferred embodiment, all or part of the conductive oligomer is synthesized first (generally with a functional group on the end for attachment to the electrode), which is then attached to the nucleoside. Additional nucleosides are then added as required, with the last step generally being attachment to the electrode. Alternatively, oligomer units are added one at a time to the nucleoside, with addition of additional nucleosides and attachment to the electrode.

10

5

A general outline of a preferred embodiment is depicted in Figure 1, using a phenyl-acetylene oligomer as generally depicted in Structure 5. Other conductive oligomers will be made using similar techniques, such as heterooligomers, or as known in the art. Thus, for example, conductive oligomers using alkene or acetylene bonds are made as is known in the art.

15

The conductive oligomer is then attached to a nucleoside that may contain one (or more) of the oligomer units, attached as depicted herein.

20

In a preferred embodiment, attachment is to a ribose of the ribose-phosphate backbone. Thus, Figure 1 depicts attachment via an amide linkage, and Figures 2 and 16 depict the synthesis of compounds with amine linkages. In a preferred embodiment, there is at least a methylene group or other short aliphatic alkyl groups (as a Z group) between the nitrogen attached to the ribose and the aromatic ring of the conductive oligomer. A representative synthesis is shown in Figure 16.

25

Alternatively, attachment is via a phosphate of the ribose-phosphate backbone. Examples of two synthetic schemes are shown in Figure 4 (synthesis of Structure 16 type compounds) and Figure 5 (synthesis of Structure 16 type compounds). Although both Figures show attachment at the 3' position of the ribose, attachment can also be made via the 2' position. In Figure 5, Z is an ethylene linker, although other linkers may be used as well, as will be appreciated by those in the art.

30

35

In a preferred embodiment, attachment is via the base. A general scheme is depicted in Figure 3, using uridine as the nucleoside and a phenylene-acetylene conductive oligomer. As will be appreciated in the art, amide linkages are also possible, such as depicted in Structure 12, using techniques well known in the art. In a preferred embodiment, protecting groups may be added to the base prior to addition of the conductive oligomers, as is generally outlined in Figures 18 and 19. In

10

15

20

25

30

35



-45-

addition, the palladium cross-coupling reactions may be altered to prevent dimerization problems; i.e. two conductive oligomers dimerizing, rather than coupling to the base.

Alternatively, attachment to the base may be done by making the nucleoside with one unit of the oligomer, followed by the addition of others.

Once the modified nucleosides are prepared, protected and activated, prior to attachment to the electrode, they may be incorporated into a growing oligonucleotide by standard synthetic techniques (Gait, Oligonucleotide Synthesis: A Practical Approach, IRL Press, Oxford, UK 1984; Eckstein) in several ways. In one embodiment, one or more modified nucleosides are converted to the triphosphate form and incorporated into a growing oligonucleotide chain by using standard molecular biology techniques such as with the use of the enzyme DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, Taq DNA polymerase, reverse transcriptase, and RNA polymerases. For the incorporation of a 3' modified nucleoside to a nucleic acid, terminal deoxynucleotidyltransferase may be used. (Ratliff, Terminal deoxynucleotidyltransferase. In The Enzymes, Vol 14A. P.D. Boyer ed. pp 105-118. Academic Press, San Diego, CA. 1981). Alternatively, and preferably, the amino nucleoside is converted to the phosphoramidite or H-phosphonate form, which are then used in solid-phase or solution syntheses of oligonucleotides. In this way the modified nucleoside, either for attachment at the ribose (i.e. amino- or thiol-modified nucleosides) or the base, is incorporated into the oligonucleotide at either an internal position or the 5' terminus. This is generally done in one of two ways. First, the 5' position of the ribose is protected with 4',4-dimethoxytrityl (DMT) followed by reaction with either 2-cyanoethoxy-bis-diisopropylaminophosphine in the presence of diisopropylammonium tetrazolide, or by reaction with chlorodiisopropylamino 2'cyanoethyoxyphosphine, to give the phosphoramidite as is known in the art; although other techniques may be used as will be appreciated by those in the art. See Gait, supra: Caruthers, Science 230:281 (1985), both of which are expressly incorporated herein by reference.

For attachment of an electron transfer moiety to the 3' terminus, a preferred method utilizes the attachment of the modified nucleoside to controlled pore glass (CPG) or other oligomeric supports. In this embodiment, the modified nucleoside is protected at the 5' end with DMT, and then reacted with succinic anhydride with activation. The resulting succinyl compound is attached to CPG or other oligomeric supports as is known in the art. Further phosphoramidite nucleosides are added, either modified or not, to the 5' end after deprotection. Thus, the present invention provides conductive oligomers covalently attached to nucleosides attached to solid oligomeric supports such as CPG, and phosphoramidite derivatives of the nucleosides of the invention

The growing nucleic acid chain may also comprise at least one nucleoside with covalently attached second electron transfer moiety. As described herein, modified nucleosides with covalently attached second electron transfer moieties may be made, and incorporated into the nucleic acid as outlined above for the conductive oligomer-nucleosides. When a transition metal complex is used as the second electron transfer moiety, synthesis may occur in several ways. In a preferred embodiment, the ligand(s) are added to a nucleoside, followed by the transition metal ion, and then the nucleoside with the transition metal complex attached is added to an oligonucleotide, i.e. by addition to the nucleic acid synthesizer. Alternatively, the ligand(s) may be attached, followed by incorportation into a growing oligonucleotide chain, followed by the addition of the metal ion.

10

15

5

In a preferred embodiment, electron transfer moieties are attached to a ribose of the ribose-phosphate backbone. This is generally done as is outlined in PCT publication SO 95/15971, using aminomodified nucleosides, at either the 2' or 3' position of the ribose. The amino group may then be used either as a ligand, for example as a transition metal ligand for attachment of the metal ion, or as a chemically functional group that can be used for attachment of other ligands or organic electron transfer moieties, for example via amide linkages, as will be appreciated by those in the art. For example, the examples describe the synthesis of a nucleoside with a metallocene linked via an amide bond to the ribose.

20

In a preferred embodiment, electron transfer moieties are attached to a phosphate of the ribose-phosphate backbone. As outlined herein, this may be done using phosphodiester analogs such as phosphoramidite bonds, see generally PCT publication WO 95/15971, or can be done in a similar manner to that depicted in Figures 4 and 5, where the conductive oligomer is replaced by a transition metal ligand or complex or an organic electron transfer moiety.

25

Attachment to alternate backbones, for example peptide nucleic acids or alternate phosphate linkages will be done as will be appreciated by those in the art.

30

35

In a preferred embodiment, electron transfer moieties are attached to a base of the nucleoside. This may be done in a variety of ways. In one embodiment, amino groups of the base, either naturally occurring or added as is described herein (see the fligures, for example), are used either as ligands for transition metal complexes or as a chemically functional group that can be used to add other ligands, for example via an amide linkage, or organic electron transfer moieties. This is done as will be appreciated by those in the art. Alternatively, nucleosides containing halogen atoms attached to the heterocyclic ring are commercially available. Acetylene linked ligands may be added using the halogenated bases, as is generally known; see for example, Tzalis et al., Tetrahedron Lett.

36(34):6017-6020 (1995); Tzalis et al., Tetrahedron Lett. 36(2):3489-3490 (1995); and Tzalis et al., Chem. Communications (in press) 1996, all of which are hereby expressly incorporated by reference. See also the examples, which describes the synthesis of a metallocene attached via an acetylene linkage to the base.

5

In one embodiment, the nucleosides are made with transition metal ligands, incorporated into a nucleic acid, and then the transition metal ion and any remaining necessary ligands are added as is known in the art. In an alternative embodiment, the transition metal ion and additional ligands are added prior to incorporation into the nucleic acid.

10

In some embodiments, as outlined herein, conductive oligomers are used between the second electron transfer moieties and the nucleosides. These are made using the techniques described herein, with the addition of the terminal second electron transfer moiety.

15

Once the nucleic acids of the invention are made, with a covalently attached conductive oligomer and optionally a second electron transfer moiety, the conductive oligomer is attached to the electrode. The method will vary depending on the type of electrode used. As is described herein, the conductive oligomers are generally made with a terminal "A" linker to facilitate attachment to the electrode. For the purposes of this application, a sulfur-gold attachment is considered a covalent attachment.

20

In a preferred embodiment, conductive oligomers are covalently attached via sulfur linkages to the electrode. However, surprisingly, traditional protecting groups for use of attaching molecules to gold electrodes are generally ideal for use in both synthesis of the compositions described herein and inclusion in oligonucleotide synthetic reactions. Accordingly, the present invention provides novel methods for the attachment of conductive oligomers to gold electrodes, utilizing unusual protecting groups, including ethylpyridine, and trimethylsilylethyl as is depicted in the Figures.

25

This may be done in several ways. In a preferred embodiment, the subunit of the conductive oligomer which contains the sulfur atom for attachment to the electrode is protected with an ethyl-pyridine or trimethylsilylethyl group. For the former, this is generally done by contacting the subunit containing the sulfur atom (preferably in the form of a sulfhydryl) with a vinyl pyridine group or vinyl trimethylsilylethyl group under conditions whereby an ethylpyridine group or trimethylsilylethyl group is added to the sulfur atom.

35

30

This subunit also generally contains a functional moiety for attachment of additional subunits, and thus additional subunits are attached to form the conductive oligomer. The conductive oligomer is then



-48-

attached to a nucleoside, and additional nucleosides attached. The protecting group is then removed and the sulfur-gold covalent attachment is made. Alternatively, all or part of the conductive oligomer is made, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. The conductive oligomer is then attached to a nucleoside, and additional nucleosides attached. Alternatively, the conductive oligomer attached to a nucleic acid is made, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. Alternatively, the ethyl pyridine protecting group may be used as above, but removed after one or more steps and replaced with a standard protecting group like a disulfide. Thus, the ethyl pyridine or trimethylsilylethyl group may serve as the protecting group for some of the synthetic reactions, and then removed and replaced with a traditional protecting group.

By "subunit" of a conductive polymer herein is meant at least the moiety of the conductive oligomer to which the sulfur atom is attached, although additional atoms may be present, including either functional groups which allow the addition of additional components of the conductive oligomer, or additional components of the conductive oligomer er. Thus, for example, when Structure 2 oligomers are used, a subunit comprises at least the first Y group.

A preferred method comprises 1) adding an ethyl pyridine or trimethylsilylethyl protecting group to a sulfur atom attached to a first subunit of a conductive oligomer, generally done by adding a vinyl pyridine or trimethylsilylethyl group to a sulfhydryl; 2) adding additional subunits to form the conductive oligomer; 3) adding at least a first nucleoside to the conductive oligomer; 4) adding additional nucleosides to the first nucleoside to form a nucleic acid; 5) attaching the conductive oligomer to the gold electrode. This may also be done in the absence of nucleosides, as is described in the Examples.

25

30

35

20

5

10

15

The above method may also be used to attach passivation molecules to a gold electrode.

In a preferred embodiment, a monolayer of passivation agents is added to the electrode. Generally, the chemistry of addition is similar to or the same as the addition of conductive oligomers to the electrode, i.e. using a sulfur atom for attachment to a gold electrode, etc. Compositions comprising monolayers in addition to the conductive oligomers covalently attached to nucleic acids (with or without second electron transfer moieties) may be made in at least one of five ways: (1) addition of the monolayer, followed by subsequent addition of the conductive oligomer-nucleic acid complex; (2) addition of the conductive oligomer-nucleic acid complex followed by addition of the monolayer; (3) simultaneous addition of the monolayer and conductive oligomer-nucleic acid complex; (4) formation of a monolayer (using any of 1, 2 or 3) which includes conductive oligomers which terminate in a

10

15

20

25

30

35

functional moiety suitable for attachment of a completed nucleic acid; or (5) formation of a monolayer which includes conductive oligomers which terminate in a functional moiety suitable for nucleic acid synthesis, i.e. the nucleic acid is synthesized on the surface of the monolayer as is known in the art. Such suitable functional moieties include, but are not limited to, nucleosides, amino groups, carboxyl groups, protected sulfur moieties, or hydroxyl groups for phosphoramidite additions. The examples describe the formation of a monolayer on a gold electrode using the preferred method (1).

In a preferred embodiment, the nucleic acid is a peptide nucleic acid or analog. In this embodiment, the invention provides peptide nucleic acids with at least one covalently attached chemical substituent. By "chemical substituent" herein is meant any chemical or biological moiety. Preferred chemical substituents include, but are not limited to, chemical functional moieties such as amino groups, thiol groups, carbon atoms, etc., which can be used to attach other moieties; labels; signaling moieties which can be used for detection; etc. Accordingly, chemical substitutents include, but are not limited to, electron transfer moieties, including electrodes, transition metal complexes, and organic electron transfer moieties; other transition metal complexes; other labels including fluoroscent labels, radioisotope labels and chemiluminescent labels; haptens such as biotin, avidin, and digoxigenin; antigens; proteins such as antibodies, ligands, receptors, and enzymes; conductive oligomers and other polymers; and other components of binding pairs such as nucleic acids.

In a preferred embodiment, the chemical substituents are covalently attached to an monomeric subunit of the PNA. By "monomeric subunit of PNA" herein is meant the -NH-CH₂CH₂-N(COCH₂-Base)-CH₂-CO- monomer, or derivatives (herein included within the definition of "nucleoside") of PNA. For example, the number of carbon atoms in the PNA backbone may be altered; see generally Nielsen et al., Chem. Soc. Rev. 1997 page 73, which discloses a number of PNA derivatives, herein expressly incorporated by reference. Similarly, the amide bond linking the base to the backbone may be altered; phosphoramide and sulfuramide bonds may be used.

In a preferred embodiment, the chemical substituents are attached to an internal monomeric subunit. By "internal" herein is meant that the monomeric subunit is not either the N-terminal monomeric subunit or the C-terminal monomeric subunit.

In this embodiment, the chemical substituents can be attached either to a base or to the backbone of the monomeric subunit. In a preferred embodiment, at least one chemical substituent is attached to an internal base. Attachment to the base is done as outlined herein or known in the literature. In general, the chemical substituents are added to a base which is then incorporated into a PNA as outlined herein. The base may be either protected, as required for incorporation into the PNA synthetic

10

15

20

25

30

35

reaction, or derivatized, to allow incorporation, either prior to the addition of the chemical substituent or afterwards. Protection and derivatization of the bases is shown in Figures 24-27. The bases can then be incorporated into monomeric subunits as shown in Figure 28. Figures 29 and 30 depict two different chemical substituents, an electron transfer moiety and a conductive oligomer, attached at a base. Figure 29 depicts a representative synthesis of a PNA monomeric subunit with a ferrocene attached to a uracil base. Figure 30 depicts the synthesis of a three unit conductive oligomer attached to a uracil base.

In a preferred embodiment, the chemical substituents are covalently attached to the backbone of the PNA monomer. The attachment is generally to one of the unsubstituted carbon atoms of the monomeric subunit, preferably the α -carbon of the backbone, as is depicted in Figures 31 and 32, although attachment at either of the carbon 1 or 2 positions, or the α -carbon of the amide bond linking the base to the backbone may be done. In the case of PNA analogs, other carbons or atoms may be substituted as well. In a preferred embodiment, chemical substituents are added at the α -carbon atoms, either to a terminal monomeric subunit or an internal one.

In this embodiment, a modified monomeric subunit is synthesized with a chemical substituent, or a functional group for its attachment, and then the base is added and the modified monomer can be incorporated into a growing PNA chain. Figure 31 depicts the synthesis of a conductive oligomer covalently attached to the backbone of a PNA monomeric subunit, and Figure 32 depicts the synthesis of a ferrocene attached to the backbone of a monomeric subunit.

Once generated, the monomeric subunits with covalently attached chemical substituents are incorporated into a PNA using the techniques outlined in Will et al., Tetrahedron 51(44):12069-12082 (1995), and Vanderlaan et al., Tett. Let. 38:2249-2252 (1997), both of which are hereby expressly incorporated in their entirety. These procedures allow the addition of chemical substituents to peptide nucleic acids without destroying the chemical substituents.

In a preferred embodiment, chemical substituents other than electron transfer moieties and transition metal complexes are attached to either or both of the bases of the terminal monomeric subunits. In this embodiment, preferred chemical substituents include fluoroscent, radioisotope and chemiluminescent labels.

As will be appreciated by those in the art, electrodes may be made that have any combination of nucleic acids, conductive oligomers and passivation agents. Thus, a variety of different conductive oligomers or passivation agents may be used on a single electrode.

10

15

20

25

30

35

Once made, the compositions find use in a number of applications, as described herein.

In a preferred embodiment, the compositions of the invention are used as probes in hybridization assays to detect target sequences in a sample. The term "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

If required, the target sequence is prepared using known techniques. For example, the sample may be treated to lyse the cells, using known lysis buffers, electroporation, etc., with purification and/or amplification occurring as needed, as will be appreciated by those in the art.

The probes of the present invention are designed to be complementary to the target sequence, such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence.

A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions; see for example Maniatis et al., Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al, hereby incorporated by reference. The hybridization conditions may also vary when a non-ionic backbone, i.e. PNA is used, as is known in the art. In addition, cross-linking agents may be added after target binding to cross-link, i.e. covalently attach, the two strands of the hybridization complex.

In a preferred embodiment, single stranded nucleic acids are made which contain a first electron transfer moiety, an electrode, and at least a second electron transfer moiety. Hybridization to a target sequence forms a double stranded hybridization complex. In a hybridization complex, at least the

sequence between the nucleosides containing the electron transfer moieties is double stranded, i.e. contains stacked π-orbitals, such that upon initiation, the complex is capable of transferring at least one electron from one of the electron transfer moieties to the other. As will be appreciated by those in the art, an electrode may serve as either an electron donor or acceptor, and the choice of the second electron transfer species is made accordingly.

In an alternative embodiment, compositions comprising a) a first single stranded nucleic acid covalently attached to an electrode via a conductive oligomer and b) a second single stranded nucleic acid containing a second electron transfer moiety, are made. In this embodiment, the first single stranded nucleic acid is capable of hybridizing to a first target domain, and the second single stranded nucleic acid is capable of hybridizing to a second target domain. The terms "first target domain" and "second target domain" or grammatical equivalents herein means two portions of a target sequence within a nucleic acid which is under examination. The first target domain may be directly adjacent to the second target domain, or the first and second target domains may be separated by an intervening target domain. Preferably, there are no gaps between the domains; i.e. they are contiguous. The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the target sequence. For example, assuming a 5'-3' orientation of the complementary target sequence, the first target domain may be located either 5' to the second domain, or 3' to the second domain.

20

5

10

15

In this embodiment, the first single stranded nucleic acid is hybridized to the first target domain, and the second single stranded nucleic acid is hybridized to the second target domain to form a hybridization complex. As outlined above, the hybridization complex is then capable of transferring at least one electron between the electron transfer moieties upon initiation.

25

30

As will be appreciated by those in the art, the hybridization complex may comprise two nucleic acids, i.e. the probe, attached to the electrode, and the target, with the second electron transfer moiety being attached to either. Alternatively, the hybridization complex may comprise three nucleic acids, i.e. the first probe, attached to the electrode, a second probe, and the target nucleic acid, nth a second electron transfer moiety attached to any of them. Similarly, hybridization complexes can be made with four or more nucleic acids, etc. What is important is that stacked π-orbitals exist between the second electron transfer moiety and the nucleoside to which the electrode is attached.

35

In one embodiment, compositions comprising a) a single stranded nucleic acid covalently attached to an electrode via a conductive oligomer, and b) a target nucleic acid are made. In this embodiment, once hybridization of the target and the probe occurs, a hybridization indicator is added. Hybridization indicators serve as an electron transfer moiety that will preferentially associate with double stranded nucleic acid is added, usually reversibly, similar to the method of Millan et al., Anal. Chem. 65:2317-2323 (1993); Millan et al., Anal. Chem. 662943-2948 (1994), both of which are hereby expressly incorporated by reference. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only occurs in the presence of double stranded nucleic acid, only in the presence of target hybridization will electron transfer occur. Intercalating transition metal complex electron transfer moieties are known in the art. Similarly, major or minor groove binding moieties, such as methylene blue, may also be used in this embodiment.

10

15

5

In addition, hybridization indicators may be used in any or all of the other systems of the invention; for example, they may be added to facilitate, quench or amplify the signal generated by the system, in addition to the covalently attached electron transfer moieties. For example, it has been shown by Millan, above, that some hybridization indicators may preferentially bind to perfectly complementary double stranded nucleic acids over nucleic acids containing mismatches. This could serve to contribute additional information about the system. Similarly, electronic coupling could be increased due to hybridization indicator binding. Alternatively, quenching of the electron transfer signal could be acheived using hybridization indicators, whereby the electrons would flow between the second electron transfer moiety and the hybridization indicator, rather than the electrode.

20

25

A further embodiment utilizes compositions comprising a) a first single stranded nucleic acid covalently attached to an electrode via a conductive oligomer; b) a second single stranded nucleic acid containing a second electron transfer moiety; and c) an intervening single stranded nucleic acid, which may or may not be labelled or contain an electron transfer moiety. As generally outlined in PCT WO 95/15971, the first single stranded nucleic acid hybridizes to the first target domain, the second single stranded nucleic acid hybridizes to the second target domain, and the intervening nucleic acid hybridizes to the intervening target domain, with electron transfer upon initiation. The intervening nucleic acid may be any length, taking into consideration the parameters for the distance between the electron transfer moieties, although it may be a single nucleoside.

30

In addition, the first and second, or first, intervening and second, nucleic acids may be ligated together prior to the electron transfer reaction, using standard molecular biology techniques such as the use of a ligase.

35

In one embodiment, the compositions of the invention are used to detect mismatches in a complementary target sequence. A mismatch, whether it be a substitution, insertion or deletion of a

nucleoside or nucleosides, results in incorrect base pairing in a hybridized double helix of nucleic acid. Accordingly, if the path of an electron from an electron donor moiety to an electron acceptor moiety spans the region where the mismatch lies, the electron transfer will be reduced such that a change in the relative impedance will be seen. Therefore, in this embodiment, the electron donor moiety is attached to the nucleic acid at a 5' position from the mutation, and the electron acceptor moiety is attached at a 3' position, or vice versa.

Electron transfer is generally initiated electronically, with voltage being preferred. A potential is applied to a sample containing modified nucleic acid probes. Precise control and variations in the applied potential can be via a potentiostat and either a three electrode system (one reference, one sample and one counter electrode) or a two electrode system (one sample and one counter electrode). This allows matching of applied potential to peak electron transfer potential of the system which depends in part on the choice of electron acceptors attached to the nucleic acid and in part on the conductive oligomer used. As described herein, ferrocene is a preferred electron transfer moiety.

15

5

10

Preferably, initiation and detection is chosen to maximize the relative difference between the impedances of double stranded nucleic acid and single stranded nucleic acid systems. The efficiency of electron transfer through nucleic acid is a function of the impedance of the compound.

20

In a preferred embodiment, a co-reductant or co-oxidant (collectively, co-redoxant) is used, as an additional electron source or sink. See generally Sato et al., Bull. Chem. Soc. Jpn 66:1032 (1993); Uosaki et al., Electrochimica Acta 36:1799 (1991); and Alleman et al., J. Phys. Chem 100:17050 (1996); all of which are incorporated by reference.

25

30

35

In a preferred embodiment, an input electron source in solution is used in the initiation of electron transfer, preferably when initiation and detection are being done using DC current, and when a passivation agent monolayer is present on the electrode. This may be done in several general ways. In a preferred embodiment, an input electron source is used that has a lower or similar redox potential than the second electron transfer moiety (ETM) covalently attached to the probe nucleic acid. Thus, at voltages above the redox potential of the input electron source, both the second ETM and the input electron source are oxidized and can thus donate electrons; the ETM donates through the hybridization complex, through the conductive oligomer, to the electrode, and the input source donates to the ETM. For example, ferrocene, as a second ETM attached to the compositions of the invention as described in the examples, has a redox potential of roughly 200 mV in aqueous solution (which changes slightly depending on what the ferrocene is bound to). Ferrocyanide, an electron source, has a redox potential of roughly 200 mV as well (in aqueous solution). Accordingly, at or above

10

15

20

25

voltages of roughly 200 mV, ferrocene is converted to ferricenium, which then transfers an electron to the nucleic acid. If this nucleic acid is double stranded, transfer proceeds rapidly through the double stranded nucleic acid, through the conductive oligomer, to the electrode. Now the ferricyanide can be oxidized to transfer an electron to the ETM. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the second ETM attached to the nucleic acid. The rate of electron donation or acceptance will be limited by the rate of diffusion of the co-reductant, which in turn is affected by the concentration and size, etc.

Alternatively, input electron sources that have lower redox potentials than the second ETM are used. At voltages less than the redox potential of the ETM, but higher than the redox potential of the electron source, the input source such as ferrocyanide is unable to be oxided and thus is unable to donate an electron to the ETM; i.e. no electron transfer occurs. The use of electron source molecules, however, is only possible when an insulating or passivation layer is present, since otherwise the source molecule will transfer electrons directly to the electrode. Accordingly, in a preferred embodiment, an electron source is used in solution to amplify the signal generated in the presence of hybridized target sequence.

In an alternate preferred embodiment, an input electron source is used that has a higher redox potential than the second electron transfer moiety (ETM) covalently attached to the probe nucleic acid. For example, luminol, an electron source, has a redox potential of roughly 720 mV. At voltages higher than the redox potential of the ETM, but lower than the redox potential of the electron source, i.e. 200 - 720 mV, the ferrocene is oxided, and transfers a single electron to the electrode via the conductive oligomer. However, the ETM is unable to accept any electrons from the luminol electron source, since the voltages are less than the redox potential of the luminol. However, at or above the redox potential of luminol, the luminol then transfers an electron to the ETM, allowing rapid and repeated electron transfer. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the second ETM attached to the nucleic acid.

30

35

Luminol has the added benefit of becoming a chemiluminiscent species upon oxidation (see Jirka et al., Analytica Chimica Acta 284:345 (1993)), thus allowing photo-detection of electron transfer through double-stranded nucleic acid. Thus, as long as the luminol is unable to contact the electrode directly. i.e. in the presence of a passivation layer, luminol can only be oxidized by transferring an electron to the second electron transfer moiety on the nucleic acid (e.g. ferrocene). When double stranded nucleic acid is not present, i.e. when the target sequence is not hybridized to the composition of the

10

15

20

25

30

35

invention, the system has a high impedance, resulting in a low photon emission and thus a low (if any) signal from the luminol. In the presence of double stranded nucleic acid, i.e. target sequence hybridization, the second electron transfer moieties have low impedance, thus generating a much larger signal. Thus, the measure of luminol oxidation by photon emission is an indirect measurement of the ability of the second electron transfer moiety to donate electrons to the electrode. Furthermore, since photon detection is generally more sensitive than electronic detection, the sensitivity of the system may be increased. Initial results suggest that luminescence may depend on hydrogen peroxide concentration, pH, and luminol concentration, the latter of which appears to be non-linear.

Suitable electron source molecules are well known in the art, and include, but are not limited to, ferricyanide, and luminol.

Alternatively, output electron acceptors or sinks could be used, i.e. the above reactions could be run in reverse, with the ETM such as a metallocene receiving an electron from the electrode, convecting it to the metallicenium, with the output electron acceptor then accepting the electron rapidly and repeatedly. In this embodiment, cobalticenium is the preferred ETM.

Electron transfer through nucleic acid can be detected in a variety of ways. A variety of detection methods may be used, including, but not limited to, optical detection, which includes fluorescence, phosphorescence, luminiscence, chemiluminescence, electrochemiluminescence, and refractive index; and electronic detection, including, but not limited to, amperommetry, voltammetry, capacitance and impedence. These methods include time or frequency dependent methods based on AC or DC currents, pulsed methods, lock-in techniques, filtering (high pass, low pass, band pass), and time-resolved techniques including time-resolved fluoroscence. In some embodiments, all that is required is electron transfer detection; in others, the rate of electron transfer may be determined.

In one embodiment, the efficient transfer of electrons from one end of a nucleic acid double helix to the other results in stereotyped changes in the redox state of both the electron donor and acceptor. With many electron transfer moieties including the complexes of ruthenium containing bipyridine, pyridine and imidazole rings, these changes in redox state are associated with changes in spectral properties. Significant differences in absorbance are observed between reduced and oxidized states for these molecules. See for example Fabbrizzi et al., Chem. Soc. Rev. 1995 pp197-202). These differences can be monitored using a spectrophotometer or simple photomultiplier tube device.

In this embodiment, possible electron donors and acceptors include all the derivatives listed above for photoactivation or initiation. Preferred electron donors and acceptors have characteristically large

spectral changes upon oxidation and reduction resulting in highly sensitive monitoring of electron transfer. Such examples include Ru(NH₃)₄py and Ru(bpy)₂im as preferred examples. It should be understood that only the donor or acceptor that is being monitored by absorbance need have ideal spectral characteristics. That is, the electron acceptor can be optically invisible if only the electron donor is monitored for absorbance changes.

In a preferred embodiment, the electron transfer is detected fluorometrically. Numerous transition metal complexes, including those of ruthenium, have distinct fluorescence properties. Therefore, the change in redox state of the electron donors and electron acceptors attached to the nucleic acid can be monitored very sensitively using fluorescence, for example with Ru(4,7-biphenyl₂-phenanthroline)₃²⁺. The production of this compound can be easily measured using standard fluorescence assay techniques. For example, laser induced fluorescence can be recorded in a standard single cell fluorimeter, a flow through "on-line" fluorimeter (such as those attached to a chromatography system) or a multi-sample "plate-reader" similar to those marketed for 96-well immuno assays.

15

5

10

Alternatively, fluorescence can be measured using fiber optic sensors with nucleic acid probes in solution or attached to the fiber optic. Fluorescence is monitored using a photomultiplier tube or other light detection instrument attached to the fiber optic. The advantage of this system is the extremely small volumes of sample that can be assayed.

20

In addition, scanning fluorescence detectors such as the FluorImager sold by Molecular Dynamics are ideally suited to monitoring the fluorescence of modified nucleic acid molecules arrayed on solid surfaces. The advantage of this system is the large number of electron transfer probes that can be scanned at once using chips covered with thousands of distinct nucleic acid probes.

25

30

Many transition metal complexes display fluorescence with large Stokes shifts. Suitable examples include bis- and trisphenanthroline complexes and bis- and trisbipyridyl complexes of transition metals such as ruthenium (see Juris, A., Balzani, V., et. al. Coord. Chem. Rev., V. 84, p. 85-277, 1988). Preferred examples display efficient fluorescence (reasonably high quantum yields) as well as low reorganization energies. These include Ru(4,7-biphenyl₂-phenanthroline)₃²⁺, Ru(4,4'-diphenyl-2,2'-bipyridine)₃²⁺ and platinum complexes (see Cummings et al., J. Am. Chem. Soc. 118:1949-1960 (1996), incorporated by reference).

Alternatively, a *reduction* in fluorescence associated with hybridization can be measured using these systems. An electron transfer "donor" molecule that fluoresces readily when on single stranded nucleic acid (with an "acceptor" on the other end) will undergo a reduction in fluorescent intensity when

complementary nucleic acid binds the probe allowing efficient transfer of the excited state electron. This drop in fluorescence can be easily monitored as an indicator of the presence of a target sequence using the same methods as those above.

In a further embodiment, electrochemiluminescence is used as the basis of the electron transfer detection. With some electron transfer moieties such as Ru²*(bpy)₃, direct luminescence accompanies excited state decay. Changes in this property are associated with nucleic acid hybridization and can be monitored with a simple photomultiplier tube arrangement (see Blackburn, G. F. Clin. Chem. 37: 1534-1539 (1991); and Juris et al., supra.

10

15

5

In a preferred embodiment, electronic detection is used, including amperommetry, voltammetry, capacitance, and impedence. Suitable techniques include, but are not limited to, electrogravimetry; coulometry (including controlled potential coulometry and constant current coulometry); voltametry (cyclic voltametry, pulse voltametry (normal pulse voltametry, square wave voltametry, differential pulse voltametry, Osteryoung square wave voltametry, and coulostatic pulse techniques); stripping analysis (aniodic stripping analysis, cathiodic stripping analysis, square wave stripping voltammetry); conductance measurements (electrolytic conductance, direct analysis); time-dependent electrochemical analyses (chronoamperometry, chronopotentiometry, cyclic chronopotentiometry and amperometry, AC polography, chronogalvametry, and chronocoulometry); AC impedance measurement; capacitance measurement; AC voltametry; and photoelectrochemistry.

20

In a preferred embodiment, monitoring electron transfer through nucleic acid is via amperometric detection. This method of detection involves applying a potential (as compared to a separate reference electrode) between the nucleic acid-conjugated electrode and a reference (counter) electrode in the sample containing target genes of interest. Electron transfer of differing efficiencies is induced in samples in the presence or absence of target nucleic acid; that is, the presence or absence of the target nucleic acid alters the impedance of the nucleic acid (i.e. double stranded versus single stranded) system which can result in different currents.

30

25

The device for measuring electron transfer amperometrically involves sensitive current detection and includes a means of controlling the voltage potential, usually a potentiostat. This voltage is optimized with reference to the potential of the electron donating complex on the nucleic acid. Possible electron donating complexes include those previously mentioned with complexes of iron, osmium, platinum, cobalt, rhenium and ruthenium being preferred and complexes of iron being most preferred.

10

15

20

25

30

In a preferred embodiment, alternative electron detection modes are utilized. For example, potentiometric (or voltammetric) measurements involve non-faradaic (no net current flow) processes and are utilized traditionally in pH and other ion detectors. Similar sensors are used to monitor electron transfer through nucleic acid. In addition, other properties of insulators (such as resistance) and of conductors (such as conductivity, impedance and capicitance) could be used to monitor electron transfer through nucleic acid. Finally, any system that generates a current (such as electron transfer) also generates a small magnetic field, which may be monitored in some embodiments.

It should be understood that one benefit of the fast rates of electron transfer observed in the compositions of the invention is that time resolution can greatly ennance the signal-to-noise results of monitors based on absorbance, fluorescence and electronic current. The fast rates of electron transfer of the present invention result both in high signals and stereotyped delays between electron transfer initiation and completion. By amplifying signals of particular delays, such as through the use of pulsed initiation of electron transfer and "lock-in" amplifiers of detection, between two and four orders of magnitude improvements in signal-to-noise may the achieved.

In a preferred embodiment, electron transfer is initiated using alternating current (AC) methods. Without being bound by theory, it appears that nucleic acids, bound to an electrode, generally respond similarly to an AC voltage across a resistor and capacitor in series. Basically, any methods which enable the determination of the nature of these complexes, which act as a resistor and capacitor, can be used as the basis of detection. Surprisingly, traditional electrochemical theory, such as exemplified in Laviron et al., J. Electroanal. Chem. 97:135 (1979) and Laviron et al., J. Electroanal. Chem. 105:35 (1979), both of which are incorporated by reference, do not accurately model the systems described herein, except for very small E_{AC} (less than 10 mV) and relatively large numbers of molecules. That is, the AC current (I) is not accurately described by Laviron's equation. This may be due in part to the fact that this theory assumes an unlimited source and sink of electrons, which is not true in the present systems.

Accordingly, alternate equations were developed, using the Nernst equation and first principles to develop a model which more closely simulates the results. This was derived as follows. The Nernst equation, Equation 1 below, describes the ratio of oxidized (O) to reduced (R) molecules (number of molecules = n) at any given voltage and temperature, since not every molecule gets oxidized at the same oxidation potential.

Equation 1

$$E_{DC} = E_0 + \frac{RT}{nF} \ln \frac{[O]}{[R]}$$
 (1)

 E_{oc} is the electrode potential, E_{o} is the formal potential of the metal complex, R is the gas constant, T is the temperature in degrees Kelvin, n is the number of electrons transferred, F is faraday's constant, [O] is the concentration of oxidized molecules and [R] is the concentration of reduced molecules.

The Nernst equation can be rearral jed as shown in Equations 2 and 3:

Equation 2

$$E_{DC} - E_0 = \frac{RT}{nF} \ln \frac{[O]}{[R]}$$
 (2)

E_{DC} is the DC component of the potential.

Equation 3

10

5

$$\exp^{\frac{nF}{RT}(E_{DC}-E_0)} = \frac{[O]}{[R]}$$
 (3)

Equation 3 can be rearranged as follows, using normalization of the concentration to equal 1 for simplicity, as shown in Equations 4, 5 and 6. This requires the subsequent multiplication by the total number of molecules.

Equation 4 [O] + [R] = 1

Equation 5 [O] = 1 - [R]

Equation 6 [R] = 1 - [O]

Plugging Equation 5 and 6 into Equation 3, and the fact that nF/RT equals 38.9 V⁻¹, for n=1, gives Equations 7 and 8, which define [O] and [R], respectively:

Equation 7

25

$$[O] = \frac{\exp^{38.9(E-E_0)}}{1 + \exp^{38.9(E-E_0)}}$$
 (4)

Equation 8

$$[R] = \frac{1}{1 + \exp^{38.9 (E - E_0)}}$$
 (5)

5

Taking into consideration the generation of an AC faradaic current, the ratio of [O]/[R] at any given potential must be evaluated. At a particular E_{oc} with an applied E_{AC} , as is generally described herein, at the apex of the E_{AC} more molecules will be in the oxidized state, since the voltage on the surface is now $(E_{oc} + E_{AC})$; at the bottom, more will be reduced since the voltage is lower. Therefore, the AC current at a given E_{oc} will be dictated by both the AC and DC voltages, as well as the shape of the Nernstian curve. Specifically, if the number of oxidized molecules at the bottom of the AC cycle is subtracted from the amount at the top of the AC cycle, the total change in a given AC cycle is obtained, as is generally described by Equation 9. Dividing by 2 then gives the AC amplitude.

Equation 9

15

10

$$i_{AC} = (electrons at [E_{DC} + E_{AC}]) - (electrons at [E_{DC} - E_{AC}])$$

Equation 10 thus describes the AC current which should result:

20

$$i_{AC} = C_0 F \omega \% ([O]_{E_{DC} + E_{AC}} - [O]_{E_{DC} - E_{AC}}) (6)$$

Equation 10

As depicted in Equation 11, the total AC current will be the number of redox molecules C), times faraday's constant (F), times the AC frequency (ω), times 0.5 (to take into account the AC amplitude), times the ratios derived above in Equation 7. The AC voltage is approximated by the average, $E_{AC}2/\pi$.

Equation 11

30

25

$$\frac{C_0 F \omega}{2} \left(\frac{-\frac{2E_{AC}}{\pi} - E_0}{\frac{2E_{DC}}{\pi} - \frac{2E_{AC}}{\pi} - E_0} \right) - \frac{38.9 [E_{DC} - \frac{2E_{AC}}{\pi} - E_0]}{\frac{2E_{AC}}{\pi} - E_0}$$

$$\frac{1 + \exp \left(\frac{1}{\pi} - \frac{2E_{AC}}{\pi} - \frac{2E_$$

Using Equation 11, simulations were generated using increasing overpotential (AC voltage). Figure 22A shows one of these simulations, while Figure 22B depicts a simulation based on traditional theory.

Figures 23A and 23B depicts actual experimental data using the Fc-wire of Example 7 plotted with the simulation, and shows that the model fits the experimental data very well. In some cases the current is smaller than predicted, however this has been shown to be caused by ferrocene degradation which may be remedied in a number of ways. However, Equation 11 does not incorporate the effect of electron transfer rate nor of instrument factors. Electron transfer rate is important when the rate is close to or lower than the applied frequency. Thus, the true i_{AC} should be a function of all three, as depicted in Equation 12.

Equation 12

10

5

 $I_{AC} = f(Nernst factors)f(k_{ET})f(instrument factors)$

These equations can be used to model and predict the expected AC currents in systems which use input signals comprising both AC and DC components. As outlined above, traditional theory surprisingly does not model these systems at all, except for very low voltages.

15

20

In general, a single stranded probe nucleic acid system has a high impedance, and a double stranded nucleic acid system (i.e. probe hybridized to target to form a hybridization complex) has a lower impedance. This difference in impedance serves as the basis of a number of useful AC detection techniques, as outlined below, but as will be appreciated by those in the art, a wide number of techniques may be used. In addition, the use of AC input and output signals enables the identification of different species based on phase shifting between the AC voltage applied and the voltage or current response. Thus, AC detection gives several advantages as is generally discussed below, including an increase in sensitivity, the ability to monitor changes using phase shifting, and the ability to "filter out" background noise.

25

Accordingly, when using AC initiation and detection methods, the frequency response of the system changes as a result of hybridization to form a double-stranded nucleic acid. By "frequency response" herein is meant a modification of signals as a result of electron transfer between the electrode and the second electron transfer moiety. This modification is different depending on signal frequency. A frequency response includes AC currents at one or more frequencies, phase shifts, DC offset voltages, faradaic impedance, etc.

30

35

In a preferred embodiment, a target sequence is added to a probe single stranded nucleic acid. Preferably, the probe single stranded nucleic acid comprises a covalently attached first electron transfer moiety comprising an electrode, and a covalently attached second electron transfer moiety as described above. However, as outlined herein, it is also possible to use a variety of other

configurations in the system, including a second electron transfer moiety attached to the target nucleic acid, a second probe nucleic acid containing a second electron transfer moiety, intervening nucleic acids, etc.

In a preferred embodiment, the single stranded nucleic acid is covalently attached to the electrode via a spacer. By "spacer" herein is meant a moiety which holds the nucleic acid off the surface of the electrode. In a preferred embodiment, the spacer is a conductive oligomer as outlined herein, although suitable spacer moieties include passivation agents and insulators as outlined above. The spacer moieties may be substantially non-conductive, although preferably (but not required) is that the rate of electron transfer through the spacer is faster than the rate through single stranded nucleic acid, although substantially conductive spacers are generally preferred. In general, the length of the spacer is as outlined for conductive polymers and passivation agents. Similarly, spacer moieties are attached as is outlined above for conductive oligomers, passivation agents and insulators, for example using the same "A" linker defined herein.

15

5

10

The target sequence is added to the composition under conditions whereby the target sequence, if present, will bind to the probe single stranded nucleic acid to form a hybridization complex, as outlined above.

20

25

A first input electrical signal is then applied to the system, preferably via at least the sample electrode (containing the complexes of the invention) and the counter electrode, to initiate electron transfer between the electrode and the second electron transfer moiety. Three electrode systems may also be used, with the voltage applied to the reference and working electrodes. The first input signal comprises at least an AC component. The AC component may be of variable amplitude and frequency. Generally, for use in the present methods, the AC amplitude ranges from about 1 mV to about 1.1 V, with from about 10 mV to about 800 mV being preferred, and from about 10 mV to about 500 mV being especially preferred. The AC frequency ranges from about 0.01 Hz to about 10 MHz, with from about 1 Hz to about 1 MHz being preferred, and from about 1 Hz to about 100 kHz being especially preferred

30

Surprisingly, the use of combinations of AC and DC signals allows the differentiation between single-stranded nucleic acid and double stranded nucleic acid, as is outlined herein. In addition, signals comprised of AC and DC components also allow surprising sensitivity and signal maximization.

In a preferred embodiment, the first input signal comprises a DC component and an AC component. That is, a DC offset voltage between the sample and counter electrodes is swept through the

10

15

20

25

30

35

electrochemical potential of the second electron transfer moiety (for example, when ferrocene is used, the sweep is generally from 0 to 500 mV). The sweep is used to identify the DC voltage at which the maximum response of the system is seen. This is generally at or about the electrochemical potential of the second electron transfer moiety. Once this voltage is determined, either a sweep or one or more uniform DC offset voltages may be used. DC offset voltages of from about -1 V to about +1.1 V are preferred, with from about -500 mV to about +800 mV being especially preferred, and from about -300 mV to about 500 mV being particularly preferred. In a preferred embodiment, the DC offset voltage is not zero. On top of the DC offset voltage, an AC signal component of variable amplitude and frequency is applied. If the nucleic acid has a low enough impedance to respond to the AC perturbation, an AC current will be produced due to electron transfer between the electrode and the second electron transfer moiety.

For defined systems, it may be sufficient to apply a single input signal to differentiate between single stranded and double stranded (i.e. the presence of the target sequence) nucleic acid. Alternatively, a plurality of input signals are applied. As outlined herein, this may take a variety of forms, including using multiple frequencies, multiple DC offset voltages, or multiple AC amplitudes, or combinations of any or all of these.

Thus, in a preferred embodiment, multiple DC offset voltages are used, although as outlined above, DC voltage sweeps are preferred. This may be done at a single frequency, or at two or more frequencies.

In a preferred embodiment, the AC amplitude is varied. Without being bound by theory, it appears that increasing the amplitude increases the driving force. Thus, higher amplitudes, which result in higher overpotentials give faster rates of electron transfer. Thus, generally, the same system gives an improved response (i.e. higher output signals) at any single frequency through the use of higher overpotentials at that frequency. Thus, the amplitude may be increased at high frequencies to increase the rate of electron transfer through the system, resulting in greater sensitivity. In addition, this may be used, for example, to induce responses in slower systems such as single stranded nucleic acids for identification, calibration and/or quantification. Thus, the amount of unhybridized single stranded nucleic acid to quantify the amount of target sequence in a sample. This is quite significant to serve as an internal control of the sensor or system. This allows a measurement either prior to the addition of target or after, on the same molecules that will be used for detection, rather than rely on a similar but different control system. Thus, the actual molecules that will be used for the detection can be quantified prior to any experiment. For example, a preliminary run at 1 Hz or less, for example, will

quantify the actual number of molecules that are on the surface of the electrode. The sample can then be added, an output signal determined, and the ratio of bound/unbound molecules determined. This is a significant advantage over prior methods.

In a preferred embodiment, measurements of the system are taken at at least two separate amplitudes or overpotentials, with measurements at a plurality of amplitudes being preferred. As noted above, changes in response as a result of changes in amplitude may form the basis of identification, calibration and quantification of the system. In addition, one or more AC frequencies can be used as well.

10

15

20

5

In a preferred embodiment, the AC frequency is varied. At different frequencies, different molecules respond in different ways. As will be appreciated by those in the art, increasing the frequency generally increases the output current. However, when the frequency is greater than the rate at which electrons may travel between the electrode and the second electron transfer moiety, higher frequencies result in a loss or decrease of output signal. For example, as depicted in Figure 11, a response may be detected at 1 Hz for both single stranded nucleic acid and double stranded nucleic acid. However, at the higher frequencies, such as 200 Hz and above, the response of the single stranded nucleic acid is absent, while the response of the double stranded nucleic acid continues to increase. At some point, the frequency will be greater than the rate of electron transfer through even double-stranded nucleic acid, and then the output signal will also drop. Thus, the different frequency responses of single stranded and double stranded nucleic acids, based on the rate at which electrons may travel through the nucleic acid (i.e. the impedance of the nucleic acid), forms the basis of selective detection of double stranded nucleic acids versus single stranded nucleic acids.

25

30

In one embodiment, detection utilizes a single measurement of output signal at a single frequency. That is, the frequency response of a single stranded nucleic acid can be previously determined to be very low at a particular high frequency. Using this information, any response at a high frequency, for example such as 10 to 100 kHz, where the frequency response of the single stranded nucleic acid is very low or absent, will show the presence of the double stranded hybridization complex. That is, any response at a high frequency is characteristic of the hybridization complex. Thus, it may only be necessary to use a single input high frequency, and any frequency response is an indication that the hybridization complex is present, and thus that the target sequence is present.

35

In addition, the use of AC techniques allows the significant reduction of background signals at any single frequency due to entities other than the covalently attached nucleic acids, i.e. "locking out" or "filtering" unwanted signals. That is, the frequency response of a charge carrier or redox active

10

15

20

25

30

35

molecule in solution will be limited by its diffusion coefficient and charge transfer coefficient. Accordingly, at high frequencies, a charge carrier may not diffuse rapidly enough to transfer its charge to the electrode, and/or the charge transfer kinetics may not be fast enough. This is particularly significant in embodiments that do not utilize a passivation layer monolayer or have partial or insufficient monolayers, i.e. where the solvent is accessible to the electrode. As outlined above, in DC techniques, the presence of "holes" where the electrode is accessible to the solvent can result in solvent charge carriers "short circuiting" the system. However, using the present AC techniques, one or more frequencies can be chosen that prevent a frequency response of one or more charge carriers in solution, whether or not a monolayer is present. This is particularly significant since many biological fluids such as blood contain significant amounts of redox active molecules which can interfere with amperometric detection methods.

In a preferred embodiment, measurements of the system are taken at at least two separate frequencies, with measurements at a plurality of frequencies being preferred. A plurality of frequencies includes a scan. For example, measuring the output signal, e.g., the AC current, at a low input frequency such as 1 - 20 Hz, and comparing the response to the output signal at high frequency such as 10 - 100 kHz will show a frequency response difference between double stranded nucleic acids with fast electron transfer rates and single stranded nucleic acids with slow electron transfer rates. In a preferred embodiment, the frequency response is determined at at least two, preferably at least about five, and more preferably at least about ten frequencies.

After transmitting the input signal to initiate electron transfer, an output signal is received or detected. The presence and magnitude of the output signal will depend on the overpotential/amplitude of the input signal; the frequency of the input AC signal; the composition of the intervening medium, i.e. the impedance, between the electron transfer moieties (i.e. single stranded versus double stranded, etc.); the DC offset; the environment of the system; the nature of the second electron transfer moiety; and the solvent. At a given input signal, the presence and magnitude of the output signal will depend in general on the impedance of the medium between the two electron transfer moieties and the character of the input signal. Double stranded nucleic acids, i.e. hybridization complexes, have relatively low impedance as compared to single stranded nucleic acids, and thus result in greater output signals. However, as noted herein, single stranded nucleic acids, in the absence of the complementary target, can result in electron transfer between the electron transfer moieties. Thus, upon transmitting the input signal, comprising an AC component and a DC offset, electrons are transferred between the first electron moiety, i.e. the electrode, and the second electron moiety covalently attached to the nucleic acid, when the impedance is low enough, the frequency is in range, and the amplitude is sufficient, resulting in an output signal.

15

20

25

30

35

In a preferred embodiment, the output signal comprises an AC current. As outlined above, the magnitude of the output current will depend on a number of parameters. By varying these parameters, the system may be optimized in a number of ways.

In general, AC currents generated in the present invention range from about 1 femptoamp to about 1 milliamp, with currents from about 50 femptoamps to about 100 microamps being preferred, and from about 1 picoamp to about 1 microamp being especially preferred.

In a preferred embodiment, the output signal is phase shifted in the AC component relative to the input signal. Without being bound by theory, it appears that surprisingly, the systems of the present invention are sufficiently uniform to allow phase-shifting based detection. That is, the complex biomolecules of the invention through which electron transfer occurs react to the AC input in a homogeneous manner, similar to standard electronic components, such that a phase shift can be determined. This may serve as the basis of detection between single-stranded and double stranded nucleic acids, but more importantly, may allow the detection of mismatches, since small changes in impedance, such as would be assumed from a mismatch present in the hybridization complex, may effect the output AC phase in a greater manner than the frequency response.

The output signal is characteristic of electron transfer through the hybridization complex; that is, the output signal is characteristic of the presence of double stranded nucleic acid. In a preferred embodiment, the basis of the detection is a difference in the faradaic impedance of the system as a result of the formation of the hybridization complex. Faradaic impedance is the impedance of the system between the two electron transfer moieties, i.e. between the electrode and the second electron transfer moiety. Faradaic impedance is quite different from the bulk or dielectric impedance, which is the impedance of the bulk solution between the electrodes. Many factors may change the faradaic impedance which may not effect the bulk impedance, and vice versa. Thus, nucleic acids in this system have a certain faradaic impedance, that will depend on the distance between the electron transfer moieties, their electronic properties, and the composition of the intervening medium, among other things. Of importance in the methods of the invention is that the faradaic impedance between the electron transfer moieties is signficantly different depending on whether the intervening nucleic acid is single stranded or double stranded. Thus, the faradaic impedance of the system changes upon the formation of a hybridization complex, and it is this change which is characteristic of the hybridization complex.

Accordingly, the present invention further provides apparatus for the detection of nucleic acids using AC detection methods. The apparatus includes a test chamber which has at least a first measuring or

sample electrode, and a second measuring or counter electrode. Three electrode systems are also useful. The first and second measuring electrodes are in contact with a test sample receiving region, such that in the presence of a liquid test sample, the two electrodes may be in electrical contact.

In a preferred embodiment, the first measuring electrode comprises a single stranded nucleic acid covalently attached via a spacer, and preferably via a conductive oligomer, such as are described herein. In one embodiment, the second electron transfer moiety may be attached to the probe single stranded nucleic acid, or it may be attached to a second probe nucleic acid, the target nucleic acid, or may be added separately, for example as an intercalator. In a preferred embodiment, the second electron transfer moiety is covalently attached to the probe single stranded nucleic acid.

The apparatus further comprises an AC voltage source electrically connected to the test chamber; that is, to the measuring electrodes. Preferably, the AC voltage source is capable of delivering DC offset voltage as well.

15

25

30

35

5

10

In a preferred embodiment, the apparatus further comprises a processor capable of comparing the input signal and the output signal. The processor is coupled to the electrodes and configured to receive an output signal, and thus detect the presence of the target nucleic acid.

Thus, the compositions of the present invention may be used in a variety of research, clinical, quality control, or field testing settings.

In a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, or any of the others well known in the art.

In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly highly conserved HIV sequences. In addition, this allows direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be

detected in this way. Bacterial infections such as tuberculosis, clymidia and other sexually transmitted diseases, may also be detected.

In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid, and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*, enterotoxic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

10

5

In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the probes in an array are used for sequencing by hybridization.

15

20

The present invention also finds use as a unique methodology for the detection of mutations or mismatches in target nucleic acid sequences. As a result, if a single stranded nucleic acid containing electron transfer moieties is hybridized to a target sequence with a mutation, the resulting perturbation of the base pairing of the nucleosides will measurably affect the electron transfer rate. This is the case if the mutation is a substitution, insertion or deletion. Alternatively, two single stranded nucleic acids each with a covalently attached electron transfer species that hybridize adjacently to a target sequence may be used. Accordingly, the present invention provides for the detection of mutations in target sequences.

25

Thus, the present invention provides for extremely specific and sensitive probes, which may, in some embodiments, detect target sequences without removal of unhybridized probe. This will be useful in the generation of automated gene probe assays.

30

In an alternate embodiment the electron transfer moieties are on separate strands. In this embodiment, one single stranded nucleic acid has an electrode covalently attached via a conductive oligomer. The putative target sequences are labelled with a second electron transfer moiety as is generally described herein, i.e. by incorporating an electron transfer moiety to individual nucleosides of a PCR reaction pool. Upon hybridization of the two single-stranded nucleic acids, electron transfer is detected.

10

15

20

25

Alternatively, the compositions of the invention are useful to detect successful gene amplification in PCR, thus allowing successful PCR reactions to be an indication of the presence or absence of a target sequence. PCR may be used in this manner in several ways. For example, in one embodiment, the PCR reaction is done as is known in the art, and then added to a composition of the invention comprising the target nucleic acid with a second ETM, covalently attached to an electrode via a conductive oligomer with subsequent detection of the target sequence. Alternatively, PCR is done using nucleotides labelled with a second ETM, either in the presence of, or with subsequent addition to, an electrode with a conductive oligomer and a target nucleic acid. Binding of the PCR product containing second ETMs to the electrode composition will allow detection via electron transfer. Finally, the nucleic acid attached to the electrode via a conductive polymer may be one PCR primer, with addition of a second primer labelled with an ETM. Elongation results in double stranded nucleic acid with a second ETM and electrode covalently attached. In this way, the present invention is used for PCR detection of target sequences.

The present invention provides methods which can result in sensitive detection of nucleic acids. In a preferred embodiment, less than about 10 X 10⁶ molecules are detected, with less than about 10 X 10⁵ being preferred, less than 10 X 10⁴ being particularly preferred, less than about 10 X 10³ being especially preferred, and less than about 10 X 10² being most preferred. As will be appreciated by those in the art, this assumes a 1:1 correlation between target sequences and reporter molecules; if more than one reporter molecule (i.e. second electron transfer moeity) is used for each target sequence, the sensitivity will go up.

While the limits of detection are currently being evaluated, based on the published electron transfer rate through DNA, which is roughly 1 X 10⁶ electrons/sec/duplex for an 8 base pair separation (see Meade et al., Angw. Chem. Eng. Ed., 34:352 (1995)) and high driving forces, AC frequencies of about 100 kHz should be possible. As the preliminary results show, electron transfer through these systems is quite efficient, resulting in nearly 100 X 10³ electrons/sec, resulting in potential femptoamp sensitivity for very few molecules.

In an additional embodiment, the present invention provides novel compositions comprising metallocenes covalently attached via conductive oligomers to an electrode, such as are generally depicted in Structure 35:

PCT/US97/20014 -

Structure 35

-71-

5

Structure 35 utilizes a Structure 4 conductive oligomer, although as will be appreciated by those in the art, other conductive oligomers such as Structures 2, 3, 9 or 10 types may be used. Preferred embodiments of Structure 35 are depicted below.

10

Structure 36

15

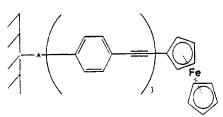
Structure 37

20

Preferred R groups of Structure 37 are hydrogen.

Structure 38

25



30

These compositions are synthesized as follows. The conductive oligomer linked to the metallocene is made as described herein; see also, Hsung et al., Organometallics 14:4808-4815 (1995); and Bumm et al., Science 271:1705 (1996), both of which are expressly incorporated herein by reference. The conductive oligomer is then attached to the electrode using the novel ethylpyridine protecting group, as outlined herein.

Once made, these compositions have unique utility in a number of applications, including photovoltaics, and infrared detection. A preferred embodiment utilizes these compounds in calibrating a potentiostat, serving as an internal electrochemistry reference in an array of the invention.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference.

10

15

20

25

5

EXAMPLES

Example 1

Synthesis of Conductive Oligomer linked via an amide to a nucleoside

This synthesis is depicted in Figure 1, using uridine as the nucleoside and a Structure 4 phenylacetylene conductive oligomer.

Compound # 1: To a solution of 10.0 gm (40 mmol) of 4-iodothioanisole in 350 mL of dichloromethane cooled in an ice-water bath was added 10.1 gm of mCPBA. The reaction mixture was stirred for half hour and the suspension was formed. To the suspension was added $4.0~\mathrm{gm}$ of powered $\mathrm{Ca}(\mathrm{OH})_2$, the mixture was stirred at room temperature for 15 min and filtered off and the solid was washed once with 30 mL of dichloromethane. To the combined filtrate was added 12 mL of trifluoroacetic anhydride and the reaction mixture was refluxed for 1.5 h under Argon. After removing the solvents, the residue was dissolved in 200 mL of a mixture of TEA and methanol (ratio = 50 : 50) and concentrated to dryness. The residue was dissolved in 100 mL of dichloromethane and the solution was washed once with 60 mL of the saturated ammonim chloride solution. The aqueous layer was extracted twice with dichloromethane (2 x 70 mL). The organic extracts were combined and dried over anhydrous sodium sulfate and immediately concentrated to dryness as quickly as possible. The residue was dissolved in 120 mL of benzene, followed by adding 5.3 mL of 4-vinylpyridine. The reaction mixture was refluxed under Argon overnight. The solvent was removed and the residue was dissolved in dichloromethane for column chromatography. Silica gel (150 gm) was packed with 20 % ethyl acetate / hexane mixture. The crude product solution was loaded and the column was eluted with 20 to 60 % ethyl acetate / hexane mixture. The fractions was identified by TLC (EtOAc : Hexane = 50 : 50, Rf = 0.24) and pooled and concentrated to dryness to afford 7.4 gm (54.2%) of the solid title compound.

35

10

15

20

25

30

35

Compound # 2: To a solution of 3.4 gm (9.97 mmol) of Compound # 1 in 70 mL of diethylamine was added 200 mg of bis(triphenylphosphine)palladium (II) chloride, 100 mg of cuprous iodide and 1.9 mL of trimethylsilylacetylene under Argon. The reaction mixture was stirred for 2 h. After removing the diethylamine, the residue was dissolved in dichloromethane for column chromatography. Silica gel (120 gm) was packed with a cosolvent of 50 % ethyl acetate / 50 % hexane. The crude sample solution was loaded and the column was eluted with the same cosolvent. After removing the solvents, the liquid title compound (2.6 gm, 83.7 %) was obtained.

Compound # 3: To a solution of 2.6 gm of Compound # 2 in 150 mL of dichloromethane colled in an ice-water bath was added 9.0 mL of 1 N tetrabutylammonium fluoride THF solution. The reaction mixture was stirred for 1 h. and washed once with water and dried over anhydrous Na₂SO₄. After removing the solvent, the residue was used for column separation. Silica gel (50 gm) was packed with a coslovent of 50 % ethyl acetate / 50 % hexane. The crude product solution was loaded and the column was eluted with the same solvents. The removal of the solvents gave the solid title compound (1.87 gm, 94.1 %).

Compound # 4: To a glass bottle were added 1.80 gm (7.52 mmol) of Compound # 3, 160 mg of bis(triphenylphosphine)palladium (II) chloride, 80 mg of cuprous iodide and 2.70 gm (9.0 mmol) of 1-trimethylsilyl-2-(4-iodophenyl)acetylene. The bottle was sealed and bubbled with Argon. Diethylamine was introduced by a syringe. The reaction mixture was heated at 50 °C under Argon for 1h. The amine was removed and the residue was dissolved in dichloromethane for the separation. Silica gel (100 gm) was packed with 60 % ethyl acetate / hexane. The crude mixture was loaded and the column was eluted with the same solvents. The fractions were identified by TLC (EtOAc: Hexane = 50:50, the product emitted blue light) and pooled. The removal of the solvents gave the solid title product (2.47 gm, 79.8 %).

Compound # 5: To a solution of 2.47 gm of Compound # 4 in 130 mL of dichloromethane cooled in an ice-water bath was added 8.0 mL of 1 N tetrabutylammonium fluoride THF solution. The reaction mixture was stirred for 1 h, and washed once with water and dried over anhydrous Na₂SO₄. After removing the solvent, the residue was used for column separation. Silica gel (60 gm) was packed with a coslovent of 50 % ethyl acetate / 50 % CH₂Cl₂. The crude solution was loaded and the column was eluted with the same solvents. The removal of solvents gave the solid title product (1.95 gm, 95.7 %).

Compound # 6: To a glass bottle were added 0.23 gm (0.68 mmol) of Compound # 5, 0.5 gm (0.64 mmol) of 2'-deoxy-2'-(4-iodophenylcarbonyl) amino-5'-O-DMT uridine, 60 mg of bis(triphenylphosphine)palladium (II) chloride, 30 mg of cuprous iodide. The bottle was sealed and

bubbled with Argon. Pyrrodine(15 mL) and DMF(15 mL) were introduced by a syringe. The reaction mixture was heated at 85 °C overnight. The solvents were removed in vacuo and the residue was dissolved in 300 mL of dichloromethane. The solution was washed three times with water and dried over sodium sulfate. After removing the solvent, the residue was subjected to column purification. Silica gel (30 gm) was packed with 1 % TEA/1 % methanol/ CH2Cl2 and the sample solution was loaded. The column was eluted with 1 % TEA/1 % methanol/ CH2Cl2 and 1 % TEA/2 % methanol/ CH2Cl2. The fractions were identified and concentrated to dryness. The separated product was subjected to another reverse-phase column purification. Reverse-phase silica gel(C-18, 120 gm) was packed with 60 % CH3CN/40 % H2O and the sample was dissolved in very small amount of THF and loaded. The column was eluted with 100 mL of 60 % CH3CN/40 % H2O, 100 mL of 70 % CH3CN/30 % H2O, 100 mL of 60 % CH3CN/10 % THF/30 % H2O, 200 mL of 50 % CH3CN/20 % THF/30 % H2O and 500 mL of 35 % CH3CN/35 % THF /30 % H2O. The fractions were identified by HPLC (0.1 mM TEAA : CH3CN = 20 : 80, flow rate = 1.0 mL/min). and concentrated to dryness to afford a pure title compound.

15

20

5

10

Compound #7: To a solution of 100 mg(0.1 mmol) of pure compound #6 in 40 mL of pyridine were added 50 mgm of DMAP and 1.0 gm (10 mmol) of succinic anhydride. The reaction mixture was stirred under Argon for 40 h. After removing pyridine, the residue was dissolved in 300 mL of dichloromethane, followed by adding 150 mL of 5 % aqueous NaHCO₃ solution. The mixture was vigorously stirred for 3 h and separated. The organic layer was washed once with 1 % citric acid solution and dried over anhydrous sodium sulfate and concentrated to dryness to give 110 mgm of Compound #7. Without further purification, the Compound #7 was used for the preparation of the corresponding CPG.

25

30

Conductive oligomer-Uridine-CPG: To 1.4 gm of LCAA-CPG(500 _) in 100 mL round bottom flask were added 110 mgm(101 µmol) of the Compound # 7, 100 mgm (230 µmol) of BOP reagent, 30 mgm (220 µmol) of HBT, 70 mL of dichloromethane and 2 mL of TEA. The mixture was shaken for three days. The CPG was filtered off and washed twice with dichloromethane and transferred into another 100 mL flask. Into CPG were added 50 mL of pyridine, 10 mL of acetic anhydride and 2mL of N-methylimidizole. The CPG was filtered off, washed twice with pyridine, methanol, dichloromethane and ether, and dried over a vacuum. The loading of the nucleoside was measured according to the standard procedure to be 7.1 µmol/gm.

35

2'-Deoxy-2'-(4-iodophenylcarbonyl)amino-5'-O-DMT uridine: To a solution of 5.1 gm(9.35 mmol) of 2'-deoxy-2'-amino-5'-O-DMT uridine in 250 mL of pyridine cooled in an ice-water bath was added 3 mL of chlorotrimethylsilane. The reaction mixture was warmed up to room temperature and stirred for

1 h. To the prepared solution were added 0.1 gm of DMAP and 3.0 gm (10.9 mmol) 4-iodobezoyl chloride and the reaction mixture was stirred overnight. To this solution was added 30 mL of concentrated ammonium hydroxide solution and the mixure was stirred for exact 15 min. The solvents were removed in vacuo. The residue was dissolved in 15 mL of dichloromethane for column separation. Silica gel (125 gm) was packed with 1 % TEA/2 % CH_3OH/CH_2Cl_2 . After loading the sample, the column was eluted with 300 mL of 1 % TEA/2 % CH_3OH/CH_2Cl_2 , and 500 mL of 1 % TEA/4 % CH_3OH/CH_2Cl_2 . The fractions were identified by TLC ($CH_3OH: CH_2Cl_2 = 10:90$) and pooled and concentrated to dryness to give 6.2 gm (85.5 %) of the pure title compound.

10 Synthesis of the Phosphormidite (Compound # 8).

To a solution of 0.2 gm of Compound # 6 and 30 mg of diisopropylammonium tetrazolide in 10 mL of dry dichloromethane is added 0.12 gm of 2-cyanoethyl N, N, N', N'-tetraisopropylphosphane under Argon. The solution was stirred for 5 h and diluted by adding 60 mL of dichloromethane. The solution was washed twice with 2.5 % w/v sodium bicarbonate solution, once with the brine and dried over sodium sulfate. After removing the solvent, residue was dissolved in 5 mL of dichloromethane, followed by adding slowly 100 mL of hexane. The suspension was stored at - 20 °C for 1 h. The supernatant was decanted and the residue was dried over a high vacuum overnight to afford 0.19 gm (79.0 %) of the title product, which will be used for DNA synthesis.

In addition, this procedure was done to make a four unit wire.

Example 2

Synthesis of conductive oligomers linked to the ribose of a nucleoside via an amine linkage

25

30

35

15

20

5

Example 2A:

Synthesis of 2'-(4-iodophenyl)amino-2'-deoxy-5'-O-DMT-uridine (Product 4): This synthesis is depicted in Figure 2, and reference is made to the labelling of the products on the figure. To a solution of 5.0 gm of 5'-O-DMT-uridine (Product 1) and 2.7 gm of dimethylaminopyridine in 200 mL of acetonitrile was added 3.3 gm of p-iodophenyl isocyalide dichloride portion by portion under Argon. The reaction mixture was stirred overnight. The mixture was diluted by adding 550 mL of dichloromethane and washed twice with 5 % sodium bicarbonate aqueous solution and once with the brine solution, and then dried over sodium sulfate. The removal of the solvent in vacuo gave the crude Product 2. Without further purification, Product 2 was dissolved in 50 mL of dry DMF and the solution was heated at 150 °C foe 2 h. After distillation of DMF, the residue was dissolved in 300 mL of dichloromethane, washed once with 5 % sodium bicarbonate solution, once with the brine solution

and dried over sodium sulfate. The removal of the solvent gave the crude **Product 3**. Without purification, the **Product 3** was dissolved 100 mL of a mixture of 50 %Dioxane and 50 % Methanol. To this solution was added 43 mL of 1N NaOH solution. The reaction mixture was stirred overnight. The mixture was diluted by adding 800 mL of dichloromethane and washed twice water and dried over Na_2SO_4 . After removing the solvent, the residue was dissolved in 15 mL of dichloromethane for the column separation. Silica gel (100 gm) of packed with 1 % TEA / 2 % Ethanol / CH_2Cl_2 , after loading the sample solution, the column was eluted with 1 % TEA / 2 - 3 % Ethanol / CH_2Cl_2 . The fractions were identified by TLC (CH3OH: CH2Cl2 = 1:9) and pooled and concentrated to give 2.0 gm (29.2 %) of the **Product 4**.

10

15

20

5

Additional conductive oligomer units can then be added to product 4 as outlined herein, with additional nucleotides added and attachment to an electrode surface as described herein.

Example 2B:

Benzylamino-uridine was synthesized as shown in Figure 16.

Synthesis of Compound C2: To a solution of 8.3 gm (15.7 mmol) of cyclonucleoside C1 in 200 mL of dichloromethane was added 2.80 gm of carbonyldiimidazole under Argon. After the solution was stirred for 7 h, into this solution were added 4.3 gm of 4-iodobenzylamine and 10 mL of diisopropylethylamine. The mixture was stirred overnight under Argon atmosphere. The solution was washed twice with 5 % Citric acid solution and dried over sodium sulfate. After concentration, the residue was dissolved in a small amount of dichgloromethane for the column separation. Silica gel (150 gm) was packed with 1 % TEA / 2 % CH_3OH / CH_2Cl_2 , upon loading the sample solution, the column was eluted with 1 % TEA / 2-10 % CH_3OH / CH_2Cl_2 . The fractions were identified by TLC ($CH_3OH : CH_2Cl_2 = 7 : 93$) and pooled and concentrated to afford 9.75 gm (78.8 %) of the product C2.

25

30

35

Synthesis of Compound C3:A mixture of 9.75 gm (12.4 mmol) of the compound C2 and 1.0 mL of DBU in 250 mL of dry THF was stirred at 50 °C under Argon for two days. THF was removed by a rotavapor and the residue was dissolved 20 mL of dichloromethane for the purification. Silica gel (130 gm) was packed with 1 % TEA / 25 % EtOAc / CH₂Cl₂, after loading the sample solution, the column was eluted with same solvent mixture. The fractions containing the desired product was pooled and concentrated to give 6.46 gm (66.3 %) of the product C3.

Synthesis of the Final Compound C4: The compound C3 (6.46 gm) was dissolved in a mixture of 150 mL of 1,4-dioxane and 100 mL of methanol, followed by adding 100 mL of 4.0 M aqueous sodium hydroxide. The mixture was stirred at room temperature overnight. The solution was diluted by adding 500 mL of dichloromethane and 500 mL of the brine solution. The mixture was shaken well

-77-

and the organic layer was separated and washed once with the 500 mL of the brine solution and dried over sodium sulfate. The dichloromethane was removed by a rotavapor and the dixoxane was removed by a high vacuum. The residue was dissolved in 20 mL of dichloromethane for the separation. Silica gel (80 gm) was packed with 1 % TEA / 25 % EtOAc / CH₂Cl₂ and the sample solution was loaded. The column was eluted with 1 % TEA / 25-50 % EtOAc / CH₂Cl₂. The right fractions were combined and concentrated to give 4.1 gm (65.7 %) of the final product C4.

Example 3

Synthesis of a conductive oligomer with an R group attached to the Y aromatic group

10

15

20

25

30

35

5

This synthesis is depicted in Figure 6.

Synthesis of 2-Acetyl-5-iodotoluene (P 1). To a suspension of 20 gm of aluminum trichloride in 500 mL of dichloremethane was added 10.2 mL of acetyl chloride under Argon. After the reaction mixture was stirred for 15 min, 3-iodotoluene (20 gm) was added through a syringe. The mixture was stirred overnight under Argon and poured into 500 gm of ice-water. Organic layer was separated and washed once with the saturated ammonium chloride solution, and washed once with 10 % sodium thiosulfate solution and dried over sodium sulfate. After removing the solvent, the residue was dissolved in hexane for the column purification. Silica gel (260 gm) was packed with hexane, after loading the sample solution, the column was eluted with 750 mL of hexane, 750 mL of 1 % v/v ether / hexane. 750 mL of 2 % v/v ether / hexane and 1500 mL of 3 % v/v ether / hexane. The fractions containing the right isomer were identified by GC-MS and ¹H NMR and pooled and concentrated to dryness to afford 12.2 gm (51.2 %) of the title product (P 1).

lodo-3-methyl-4-(ehynyl trimethylsilyl) benzene (P2). Under inert atmosphere 500 ml bound bottom flask was charged with 25 ml of dry THF, cooled to -78°C and 14 ml of 2.0 M LDA solution (heptane/ethylbenzene/ THF solution) was added by syringe. To this solution 6.34 gr (24.38 mmole) of iodo-3-methyl-4-acetyl benzene in 25 ml of THF was added dropwise and the reaction mixture was stirred for 1 hr at -78°C, then 4.0 ml (19.42 mmole) of diethylchlorophosphate were added by syringe. After 15 min cooling bath was removed and the reaction mixture was allowed to heat up to RT and stirred for 3 hrs. The resulted mixture was cooled again to -78°C and 29 ml of 2.0 M LDA solution were added dropwise. At the end of the addition the reaction mixture was allowed to warm up to RT and stirred for additional 3 hrs. After that period of time it was cooled again to -20°C, 9.0 ml (70.91 mmole) of trimethylsilyl chloride were injected and the stirring was continued for 2 hrs at RT. The reaction mixture was poured into 200 ml of ice/sodium bicarbonate saturated aqueous solution and 300 ml of ether were added to extract organic compounds. The aqueous phase was separated and

extracted again with 2x100 ml of ether. The ether fractions were combined, dried over sodium sulfate and evaporated. The resulted liquid residue was purified by silica gel chromatography (100% n-hexane as eluent). 4.1 gr (54% yield) were obtained.

Synthesis of Product (P 3). To a solution of 1.14 gm of Compound # 3 (as described above) and 1.60 gm of P 2 in 100 mL of diethylamine were added 0.23 gm of $\{1,1'-$ bis(diphenylphosphino)ferrocene]palladium (II) chloride and 0.1 gm of copper (I) iodide under Argon. The reaction mixture was stirred at 55 °C for 1 h and stirred at room temperature overnight. After removing the solvent, the residue was dissolved in dichloromethane for column separation. Silica gel (120 gm) was packed with 20 % ethyl acetate / CH_2CI_2 . The sample solution was loaded and the column was eluted with 20 - 50 % ethyl acetate / CH_2CI_2 . The fractions were identified by TLC (EtOAC: $CH_2CI_2 = 50$: 50) and pooled and concentrated to give 1.70 gm (84.0 %) of TMS-derivative of P 3.

To a solution of 0.74 gm of TMS-derivative of P 3 in 70 mL of dichloromethane at 0 °C was added 2.2 mL of 1.0 M (nBu)₄NF THF solution. After stirring for 30 min, the solution was washed once with water and dried over sodium sulfate. The solvent was removed, the residue was used for column separation. Silica gel (20 gm) was packed with 20 % ethyl acetate / CH_2Cl_2 , the column was eluted with 20 - 40 % ethyl acetate / CH_2Cl_2 . The fractions containing the fluorescent compound were combined and concentrated to dryness to afford 0.5 gm (81.3 %) of the pure P 3.

20

25

5

10

15

Synthesis of P 4: To a solution of 0.5 gm of P 3 and 0.63 gm of P 2 in 50 mL of dry DMF and 10 mL of TEA were added 100 mgm of [1,1]-bis(diphenylphosphino)ferrocene]palladium (II) chloride and 50 mgm of copper (I) rodide under Argon. The reaction mixture was stirred at 55 °C for 1 h and stirred at 35 °C overnight. The solvents were removed in vacuo and the residue was dissolved in 10 mL of CH_2CI_2 for column separation. Silica gel (100 gm) was packed with 20 % ethyl acetate / CH_2CI_2 , after loading the sample, the column was eluted with 20 - 40 % ethyl acetate / CH_2CI_2 . The fractions were identified by TLC (EtOAC : CH_2CI_2 = 50 : 50) and pooled and concentrated to give 0.47 gm (61.3 %) of TMS-derivative of P 4.

To a solution of 0.47 gm of TMS-derivative of P 4 in 70 mL of dichloromethane at 0 °C was added 1.0 mL of 1.0 M (nBu)₄NF THF solution. After stirring for 30 min, the solution was washed once with water and dried over sodium sulfate. The solvent was removed, the residue was used for column separation. Silica gel (20 gm) was packed with 20 % ethyl acetate / CH₂Cl₂, the column was eluted with 20 - 40 % ethyl acetate / CH₂Cl₂. The fractions containing the fluorescent compound were

combined and concentrated to dryness to afford 0.32 gm (78.7 %) of the pure P 4.

Other conductive oligomers with R groups are depicted in Figure 17, which were made using the techniques outlined herein.

Example 4

5

10

15

25

30

35

Synthesis of a nucleoside with a metallocene second electron transfer moiety attached via a ribose

Synthesis of 5'-O-DMT-2'-deoxy-2'-(ferrocenecarbonyl)amino Uridine (UAF): To a solution of 2.5 gm(10.9 mmol) of ferrocene monocarboxylic acid in 350 mL of dichloromethane were added 2.25 gm (10.9 mmol) of DCC and 1.27 gm (10.9 mmol) of N-hydroxysuccinimide. The reaction mixture was stirred for 3 h and the precipitate was formed. The precipitate was filtered off and washed once with dichloromethane. The combined filtrate was added into 4.5 gm (8.25 mmol) of 2'-deoxy-2'-amino-5'-O-DMT uridine, followed by adding 2 mL of triethylamine. The reaction mixture was stirred at room temperature for 8 days. After removing the solvent, the residue was dissolved in dichloromethane for separation. Silica gel (120 gm) was packed with 1 % TEA / 2 % CH_3OH / CH_2Cl_2 . After loading the sample solution, the column was eluted with 2-7 % CH_3OH /1 % TEA / CH_2Cl_2 . The fraction was identified by $TLC(CH_3OH : CH_2Cl_2 = 1 : 9)$ and pooled and concentrated to dryness to afford 1.3 gm(22.0 %) of the title compound.

20 Synthesis of UAF Phosphoramidite:

Preparation of Diisopropylaminochloro(β-cyano)ethoxyphosphine: To a solution of 0.54 mL(4.0 mmol) of dichloro(β-cyano)ethoxyphosphine in 40 mL of dichloromethane cooled in an ice-water bath was added 10 mL of diisopropylethylamine, followed by adding 0.64 mL (4.0 mmol) of diisopropylamine under Argon. The reaction mixture was warmed up to room temperature and stirred for 2 h. After adding 0.1 gm of DMAP into the solution, the reaction mixture is ready for the next step reaction.

Preparation of **UAF phosphoramidite**: To a solution of 1.30 gm (1.72 mmol) of 5'-O-DMT-5-ferrocenylacetylenyl-2'-deoxy uridine in 40 mL of dichloromethane cooled in an ice-water bath was added 10 mL of diisopropylethylamine. The prepared phosphine solution was transferred into the nucleoside solution through a syringe. The reaction mixture was warmed up to room temperature and stirred overnight. The solution was diluted by adding 100 mL of dichloromethane and washed once with 200 mL od 5 % aqueous NaHCO₃ solution, and once with the brine (200 mL) and dried over Na₂SO₄ and concentrated to dryness. Silica gel(47 gm) was packed with 2 % TEA/1 % CH₃OH/CH₂Cl₂. The residue was dissolved in 10 mL of dichloromethane and loaded. The column

10

15

20

25

30

was eluted with 150 mL of 1 % TEA / 1 % CH₃OH / CH₂Cl₂ and 250 mL of 1 % TEA /2 % CH₃OH / CH₂Cl₃. The fractions were pooled and concentrated to give 0.5 gm (30.3 %) of the title compound.

Nucleotides containing conductive oligomers and second electron transfer moieties were incorporated into nucleic acids using standard nucleic acid synthesis techniques; see "Oligonucleotides and Analogs, A Practical Approach". Ed. By F. Eckstein, Oxford University Press, 1991, hereby incorporated by reference.

Example 5

Synthesis of a nucleoside with a metallocene second electron transfer moiety attached via the base

Synthesis of 5'-O-DMT-5-ferrocenylacetylenyl-2'-deoxy uridine (UBF): In a flask were added 4.8 gm(13.6 mmol) of 5-iodo-2'-deoxy uridine, 400 mg of bis(triphenylphosphine)palladium (II) chloride, 100 mg of cuprous iodide, 95 mL of DMF and 10 mL of TEA. The solution was degassed by Argon and the flask was sealed. The reaction mixture was stirred at 50 °C overnight. After removing solvents in vacuo, the residue was dissolved in 140 mL of dry pyridine, followed by adding 0.2 gm of DMAP and 5.0 gm (14.8 mmol) of DMT-Cl. The reaction mixture was stirred at RT overnight. After removing the solvent, the residue was dissolved in 300 mL of dichloromethane and washed twice with 5 % aqueous NaHCO₃ (2 x 200 mL), twice with the brine (2 x 200 mL) and dried over sodium sulfate. The solvent was removed and the residue was coevaporated twice with toluene and dissolved in 15 mL of dichloromethane for column separation. Silica gel (264 gm) was packed 0.5 % TEA/CH₂Cl₂ After loading the crude product solution, the column was eluted with 300 mL of 1 % TEA/2 % CH₃OH/CH₂Cl₂, 400 mL of 1 % TEA/5 % CH₃OH/CH₂Cl₂, and 1.2 L of 1 % TEA/7 % CH₃OH/CH₂Cl₂. The fractions were identified by TLC(CH₃OH : CH₂Cl₂ = 10 : 90) and pooled and concentrated to dryness to give 7.16 gm (71.3 %) of the title compound.

Synthesis of UBF Phosphoramidite:

Preparation of Diisopropylaminochloro(β-cyano)ethoxyphosphine: To a solution of 1.9 mL(13.8 mmol) of dichloro(β-cyano)ethoxyphosphine in 40 mL of dichloromethane cooled in an ice-water bath was added 10 mL of diisopropylethylamine, followed by adding 2.3 mL (13.8 mmol) of diisopropylamine under Argon. The reaction mixture was warmed up to room temperature and stirred for 2 h. After adding 0.1 gm of DMAP into the solution, the reaction mixture is ready for next step reaction.

Preparation of UBF phosphoramidite: To a solution of 3.42 gm (4.63 mmol) of 5'-O-DMT-5-ferrocenylacetylenyl-2'-deoxy uridine in 40 mL of dichloromethane cooled in an ice-water bath was added 10 mL of diisopropylethylamine. The prepared phosphine solution was transferred into the nucleoside solution through a syringe. The reaction mixture was warmed up to room temperature and stirred overnight. The solution was diluted by adding 150 mL of dichloromethane and washed once with 200 mL of 5 % aqueous NaHCO₃ solution, and once with the brine (200 mL) and dried over Na₂SO₄ and concentrated to dryness. Silica gel(92 gm) was packed with 2 % TEA/1 % CH₃OH/CH₂Cl₂. The residue was dissolved in 10 mL of dichloromethane and loaded. The column was eluted with 500 mL of 1 % TEA/2 % CH₃OH/CH₂Cl₂. The fractions were pooled and concentrated to give 3.0 gm (69.0 %) of the title compound.

Nucleotides containing conductive oligomers and second electron transfer moieties were incorporated into nucleic acids using standard nucleic acid synthesis techniques; see "Oligonucleotides and Analogs, A Practical Approach", Ed. By F. Eckstein, Oxford University Press, 1991, hereby incorporated by reference.

Example 6

Synthesis of an electrode containing nucleic acids containing conductive oligomers with a monolayer of (CH₂)₁₆

20

30

35

15

5

10

Using the above techniques, and standard nucleic acid synthesis, the uridine with the phenylacetylene conductive polymer of Example 1 was incorporated at the 3' position to form the following nucleic acid: ACCATGGACTCAGCU-conductive polymer of Example 1 (hereinafter "wire-1").

25 HS-(CH2)16-OH (herein "insulator-2") was made as follows.

16-Bromohexadecanoic acid. 16-Bromohexadecanoic acid was prepared by refluxing for 48 hrs 5.0 gr (18.35 mmole) of 16-hydroxynexadecanoic acid in 24 ml of 1:1 v/v mixture of HBr (48% aqueous solution) and glacial acetic acid. Upon cooling, crude product was solidified inside the reaction vessel. It was filtered out and washed with 3x100 ml of cold water. Material was purified by recrystalization from n-hexane, filtered out and dried on high vacuum. 6.1 gr (99% yield) of the desired product were obtained.

16-Mercaptohexadecanoic acid. Under inert atmosphere 2.0 gr of sodium metal suspension (40% in mineral oil) were slowly added to 100 ml of dry methanol at 0°C. At the end of the addition reaction mixture was stirred for 10 min at RT and 1.75 ml (21.58 mmole) of thioacetic acid were added. After

additional 10 min of stirring, 30 ml degassed methanolic solution of 6.1 gr (18.19 mmole) of 16-bromohexadecanoic acid were added. The resulted mixture was refluxed for 15 hrs, after which, allowed to cool to RT and 50 ml of degassed 1.0 M NaOH aqueous solution were injected. Additional refluxing for 3 hrs required for reaction completion. Resulted reaction mixture was cooled with ice bath and poured, with stirring, into a vessel containing 200 ml of ice water. This mixture was titrated to pH=7 by 1.0 M HCl and extracted with 300 ml of ether. The organic layer was separated, washed with 3x150 ml of water, 150 ml of saturated NaCl aqueous solution and dried over sodium sulfate. After removal of ether material was purified by recrystalization from n-hexane, filtering out and drying over high vacuum. 5.1 gr (97% yield) of the desired product was obtained.

10

5

16-Bromohexadecan-1-ol. Under inert atmosphere 10 ml of BH₃·THF complex (1.0 M THF solution) were added to 30 ml THF solution of 2.15 gr (6.41 mmole) of 16-bromohexadecanoic acid at -20°C. Reaction mixture was stirred at this temperature for 2 hrs and then additional 1 hr at RT. After that time the resulted mixture was poured, with stirring, into a vessel containing 20° m. of ice/saturated sodium bicarbonate aqueous solution. Organic compounds were extracted with 3x200 ml of ether. The ether fractions were combined and dried over sodium sulfate. After removal of ether material was dissolved in minimum amount of dicloromethane and purified by silica gel chromatography (100% dicloromethane as eluent). 1.92 gr (93% yield) of the desired product were obtained.

20

25

15

16-Mercaptohexadecan-1-ol. Under inert atmosphere 365 mg of sodium metal suspension (40% in mineral oil) were added dropwise to 20 ml of dry methanol at 0°C. After completion of addition the reaction mixture was stirred for 10 min at RT followed by addition of 0.45 ml (6.30 mmole) of thioacetic acid. After additional 10 min of stirring 3 ml degassed methanolic solution of 1.0 gr (3.11 mmole) of 16-bromohexadecan-1-ol were added. The resulted mixture was refluxed for 15 hrs, allowed to cool to RT and 20 ml of degassed 1.0 M NaOH aqueous solution were injected. The reaction completion required additional 3 hr of reflux. Resulted reaction mixture was cooled with ice bath and poured, with stirring, into a vessel containing 200 ml of ice water. This mixture was titrated to pH=7 by 1.0 M HCl and extracted with 300 ml of ether. The organic layer was separated, washed with 3x150 ml of water, 150 ml of saturated NaCl aqueous solution and dried over sodium sulfate. After ether removal material was dissolved in minimum amount of dicloromethane and purified by silica gel chromatography (100% dicloromethane as eluent). 600 mg (70% yield) of the desired product were obtained.

30

35

A clean gold covered microscope slide was incubated in a solution containing 100 micromolar HS- $(CH_2)_{16}$ -COOH in ethanol at room temperature for 4 hours. The electrode was then rinsed throughly

10

15

20

25

with ethanol and dried. 20-30 microliters of wire-1 solution (1 micromolar in 1XSSC buffer at pH 7.5) was applied to the electrode in a round droplet. The electrode was incubated at room temperature for 4 hours in a moist chamber to minimize evaporation. The wire-1 solution was then removed from the electrode and the electrode was immersed in 1XSSC buffer followed by 4 rinses with 1XSSC. The electrode was then stored at room temperature for up to 2 days in 1XSSC.

Alternatively, and preferably, either a "two-step" or "three-step" process is used. The "two-step" procedure is as follows. The wire-1 compound, in water at ~ 5-10 micromolar concentration, was exposed to a clean gold surface and incubated for ~ 24 hrs. It was rinsed well with water and then ethanol. The gold was then exposed to a solution of ~ 100 micromolar insulator thiol in ethanol for ~ 12 hrs, and rinsed well. Hybridization was done with complement for over 3 hrs. Generally, the hybridization solution was warmed to 50°C, then cooled in order to enhance hybridization.

The "three-step" procedure uses the same concentrations and solvents as above. The clean gold electrode was incubated in insulator solution for ~ 1 hr and rinsed. This procedure presumably results in an incomplete monolayer, which has areas of unreacted gold. The slide was then incubated with wire-1 solution for over 24 hrs (generally, the longer the better). This wire-1 still had the ethyl-pyridine protecting group on it. The wire-1 solution was 5% NH4OH, 15% ethanol in water. This removed the protecting group from the wire and allowed it to bind to the gold (an in situ deprotection). The slide was then incubated in insulator again for ~ 12 hrs, and hybridized as above.

In general, a variety of solvent can be used including water, ethanol, acetonitrile, buffer, mixtures etc. Also, the input of energy such as heat or sonication appears to speed up all of the deposition processes, although it may not be necessary. Also, it seems that longer incubation periods for both steps, for example as long as a week, the better the results.

Hybridization efficiency was determined using ³²P complementary and noncomplementary 15 mers corresponding to the wire-1 sequence. The electrodes were incubated with 50 microliters of each of the labelled non-complementary (herein "A5") or complementary (herein "S5") target sequences applied over the entire electrode in 1XSSC as depicted in Table 1. The electrodes were then incubated for 1-2 hours at room temperature in a moist chamber, and rinsed as described above. The amount of radiolabelled DNA was measured for each electrode in a scintllation counter, and the electrodes were dried and exposed to X-ray film for 4 hours.

10

15

20

25

Table 1

		140.0	
hybridized with:	total ³² P counts added	³² P counts hybridized to surface	
A5, 20% specific activity, DNA concentration 1 nM, 1 hour incubation	46,446	152	
S5, 30% specific activity, DNA concentration 1 nM, 1 hour incubation	39,166	10,484 (27% hybridized)	
A5, 14% specific activity, DNA concentration 5 nM, 2 hour incubation	182,020	172	
S5, 20% specific activity, DNA concentration 5 nM, 2 hour incubation	96,284	60,908 (63% hybridized)	

Example 7
Synthesis of compositions containing ferrocene linked to an electrode

It has been shown in the literature that cyclic voltametry, and other DC techniques, can be used to determine the electron transfer rate of surface bound molecules. Surface bound molecules should show perfectly symetric oxidation and reduction peaks if the scan speed of the voltammagram is sufficiently slow. As the scan rate is increased, these peaks are split apart due to the kinetics of electron transfer through the molecules. At a given scan speed, a poorly conducting molecule should exhibit greater splitting than a good conductor. As the speed is increased, the poor conductor will be split even more.

Accordingly, to test the conductivity of the conductive polymer as compared to a traditional insulator, two molecules were tested. The synthesis of ferrocene attached via a conductive oligomer to an electrode (herein "wire-2") was made as follows, as depicted in Figure 7.

Synthesis of compound #11 was as follows. 2.33 gr (5.68 nmole) of compound #10 (made as described in Hsung et al., Organometallics 14:4808-4815 (1995), incorporated by reference), 90 mg (0.47 mmole) of CuI and 80 mg (0.11 mmole) of $PdCl_2(PPh_3)_2$ were dissolved in 100 ml of pyrrolidine under inert atmosphere and heated for 20 hrs at 50°C. All volatile components were removed on high vacuum and resulted crude residue was dissolved in minimum amount of dichloromethane. The desired compound was purified by silica gel chromatography (50% ethyl acetate + 50% dichloromethane as eluent). 3.2 gr (90% yield) of the pure product were obtained.

10

20

25

30

35

Compound #12. To 200 mg (0.32 mmole) of suspension of MG#1 in 200 ml of acetone (sonication was applied in order to get better results) 3 ml of MeI were added and the reaction mixture was stirred for 20 hrs at RT. After that time volume of the resulted solution was reduced by rotovap evaporation to 50 ml and then 400 ml of n-hexane were added. Formed precipitate was filtered out, washed with 3x200 ml of n-hexane and dried on high vacuum. Quantitative yield of the desired compound was obtained.

Compound #13. To 100 mg (0.13 mmole) of suspension of MG#2 in 200 ml of acetone (sonication was applied in order to get better results) 10 ml of triethyl amine were added and the reaction mixture was stirred for 20 hrs at RT. After that time volume of the resulted solution was reduced by rotovap evaporation to 50 ml and then 400 ml of n-hexane were added. Formed precipitate was filtered out, washed with 3x200 ml of n-hexane and dried on high vacuum. The desired compound was extracted from this precipitate with 3x50 ml of THF. Evaporation of the THF fractions gave 35 mg (52%) of the compound #13. This was then added to a gold electrode as known in the art.

HS-(CH2)15NHCO-Fc (herein "insulator-1") was imade as described in Ward et al., Anal. Chem. 66:3164-3172 (1994), hereby incorporated by reference (note: the Figure 1 data has been shown to be incorrect, although the synthesis of the molecule is correct).

Monolayers of each were made as follows. <u>Insulator</u>: Gold covered microscope slides were immersed in a mixture of insulator-1 and HS-(CH2)15-OH (insulator-2) in neat ethanol. Insulator-2 molecule is added to the mixture to prevent the local concentration of ferrocene at any position from being too high, resulting in interactions between the ferrocene molecules. The final solution was 0.1 mM insulator-1 and 0.9 mM insulator-2. The mixture was sonicated and heated (60-80°C) for 1-10 hours. The electrodes were rinsed thoroughly with ethanol, water and ethanol. The electrodes were immersed in a 1 mM thiol solution in neat ethanol and let stand at room temperature for 2-60 hours. The electrodes were then rinsed again. This procedure resulted in 1-10% coverage of insulator-1 as compared to calculated values of close packed ferrocene molecules on a surface. More or less coverage could easily be obtained by altering the mixture concentration and/or incubation times.

Wires: The same procedure was followed as above, except that the second step coating required between 10 and 60 hours, with approximately 24 hours being preferable. This resulted in lower coverages, with between 0.1 and 3% occurring.

Cyclic voltametry was run at 3 scan speeds for each compound: 1V/sec, 10 V/ec, and 50 V/sec. Even at 1 V/sec, significant splitting occurs with insulator-1, with roughly 50 mV splitting occuring. At higher

speeds, the splitting increases. With wire-2, however, perfectly symmetrical peaks are observed at the lower speeds, with only slight splitting occurring at 50 V/sec.

It should be noted that despite a significant difference in electron transfer rate, electron transfer does still occur even in poorly conducting oligomers such as (CH₂)₁₅, traditionally called "insulators". Thus the terms "conductive oligomer" and "insulator" are somewhat relative.

Example 8

Synthesis and analysis of nucleic acid with both a conductive oligomer and a second electron transfer moiety

The following nucleic acid composition was made using the techniques above: 5'ACCATGGAC[UBF]CAGCU-conductive polymer (Structure 5 type, as outlined above) herein "wire-3", with UBF made as described above. Thus, the second electron transfer moiety, ferrocene, is on the sixth base from the conductive oligomer.

Mixed monolayers of wire-3 and insulator-2 were constructed using the techniques outlined above. The compositions were analyzed in 0.2 M NaClO₄ in water using cyclic voltametry (CV) and square wave voltametry (SW), in the absence (i.e. single stranded) and presence (i.e. double stranded) of complementary target sequence.

The results of SW show the absence of a peak prior to hybridization, i.e. in the absence of double stranded nucleic acid. In the presence of the complementary target sequence, a peak at ~240 mV, corresponding to ferrocene, was seen.

A mediator as described herein was also used. 6 mM ferricyanide (Fe(CN)₆) was added to the solution. Ferricyanide should produce a peak at 170 mV in a SW experiment. However, no peak at 170 mV was observed, but the peak at 240 mV was greatly enhanced as compared to the absence of ferricyanide.

Alternatively, CV was done. No peaks were observed in the absence of target sequence. Once again, the chip was incubated with perfectly complimentary nucleic acid in order to hybridize the surface nucleic acid. Again, the chip was scanned under the same conditions. An increased signal was observed. Finally, the chip was soaked in buffer at 70°C in order to melt the compliment off the surface. Previous experiments with radioactive probes have shown that 15-mers hybridized on a very

35

5

10

15

20

similar surface melted at approximately 45°C. Repeating the scan after the heat treatment shows a reduced signal, as in the first scan prior to hybridization.

Example 9

5

AC detection methods

Electrodes containing four different compositions of the invention were made and used in AC detection methods. In general, all the electrodes were made by mixing a ratio of insulator-2 with the sample as is generally outlined above.

10

15

20

25

30

35

Sample 1, labeled herein as "Fc-alkane", contained a mixed monolayer of insulator-2 and insulator-1. Sample 2, labeled herein as "Fc-amido-alkane", contained a mixed monolayer of insulator-2 and a derivative of insulator-1 which has an amido attachment of the ferrocene to the alkane. Sample 3, labeled herein as "Fc-wire", contained a mixed monolayer of insulator-2 and wire-2. Sample 4 was the same as Sample 3, with the exception that a new in situ deprotection step was used, described below. Sample 5, labeled herein as "ssDNA" (AGCTGAGTCCA(UBF)GGU-conductive oligomer), contained a mixed monolayer of insulator-2 and wire-3. Sample 6, labeled herein as "dsDNA", contained a mixed monolayer of insulator-2 and wire-3, wherein the complement of wire-3 was hybridized to form a double stranded wire-3. Sample 7 was a solution of ferrocene in solution. As is shown herein, the rate of electron transfer, from fast to slower, is as follows: Sample 3 > Sample 6 > Sample 1 > Sample 2 > Sample 5. Generally, Sample 1 models ssDNA, and Sample 3 models dsDNA.

The experiments were run as follows. A DC offset voltage between the working (sample) electrode and the reference electrode was swept through the electrochemical potential of the ferrocene, typically from 0 to 500 mV. On top of the DC offset, an AC signal of variable amplitude and frequency was applied. The AC current at the excitation frequency was plotted versus the DC offset.

Figure 8 depicts an experiment with Sample 1, at 200 mV AC amplitude and frequencies of 1, 5 and 100 Hz. Sample 1 responds at all three frequencies, and higher currents result from higher frequencies, which is simply a result of more electrons per second being donated by the ferrocene at higher frequencies. The faster the rate, the higher the frequency response, and the better the detection limit. Figure 9 shows overlaid AC voltammograms of an electrode coated with Sample 3. Four excitation frequencies were applied: 10 Hz, 100 Hz, 1 kHz, 10 kHz, all at 25 mV overpotential. Figure 10, shows the frequency response of samples 1, 2 and 3 by measuring the peak currents vs. frequency. Sample 3 response to increasing frequencies through 10 kHz (the detector system limit), while Sample 1 lose its responses at between 20 and 200 Hz. Thus, to discriminate between Sample

5

10

15

20

25

1 and Sample 3, one could simplify the methods by analyzing it at 1 Hz and 1000 Hz and compare the responses, although as will be appreciated, this is only one method of a variety of possible methods. This should be similar to the dsDNA and ssDNA system. Figure 11 shows Sample 5 and Sample 6, plotted as a function of normalized current (with the highest current being 1 for both cases; the actual current of dsDNA is much higher than that of ssDNA, so the graph was normalized to show both). The lines are modeled RC circuits, as described above, and not a fit to the data. At 1 Hz, both ssDNA and dsDNA respond; at 200 Hz, the ssDNA signal is gone. Figure 12 shows that increasing the overpotential will increase the output signal for slow systems like samples 1 and 2. Figures 13A and 13B show that the overpotential and frequency can be tuned to increase the selectivity and sensitivity. For example, a low overpotential and high frequence can be used to minimize the slower species (Sample 1 or Sample 5). Then the overpotential can be increased to induce a response in the slower species for calibration and quantification.

Figure 14 shows that the ferrocene added to the solution (Sample 7) has a frequency response related to diffusion that is easily distinguishable from the frequency response of attached ferrocene. This indicates that by varying frequency, signals from bound molecules, particular fast bound molecules such as dsDNA, can be easily distinguished from any signal generated by contaminating redox molecules in the sample.

Figures 15A and 15B shows the phase shift that results with different samples. Figure 15A shows the model compounds, and 15B shows data with dsDNA and ssDNA. While at this frequency, the phase shift is not large, a frequency can always be found that results in a 90° shift in the phase.

Example 10

Synthesis of conductive oligomers attached via a base

Representative syntheses are depicted in Figures 18 and 19. When using palladium coupling chemistry, it appears that protecting groups are required on the base, in order to prevent significant dimerization of conductive oligomers instead of coupling to the iodinated base. In addition, changing the components of the palladium reaction may be desirable also. Also, for longer conductive oligomers, R groups are preferred to increase solubility.

Example 11

The use of trimethylsilylethyl protecting groups

35

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶:

C12Q 1/68, G01N 33/53

(11) International Publication Number:

(43) International Publication Date:

23 July 1998 (23.07.98)

WO 98/31839

(21) International Application Number:

PCT/US98/01144

A2

(22) International Filing Date:

21 January 1998 (21.01.98)

(30) Priority Data:

08/786,153 21 January 1997 (21.01.97) US 08/804,883 24 February 1997 (24.02.97) US 08/843,623 10 April 1997 (10.04.97) US

- (71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mount Auburn Street, Cambridge, MA 02138-5701 (US).
- (72) Inventor: BAMDAD, Cynthia, C.; 621 Sierra Madre Boulevard, San Marino, CA 91108 (US).
- (74) Agent: OYER, Timothy, J.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

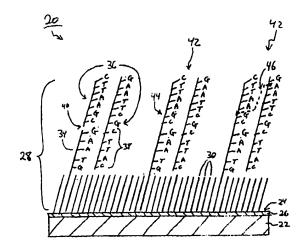
Published

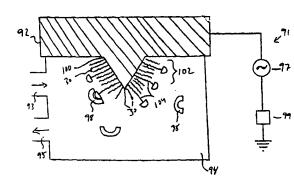
Without international search report and to be republished upon receipt of that report.

(54) Title: ELECTRONIC-PROPERTY PROBING OF BIOLOGICAL MOLECULES AT SURFACES

(57) Abstract

A technique for immobilizing biological molecules, in particular nucleic acid strands, is described. Biological molecules immobilized at surfaces can be used in electron-transfer detection techniques in which a binding partner of a biological molecule is brought into proximity of the surface-immobilized biological molecule, an electrical potential created between the two biologically-binding species, and electron transfer through the species determined. Another technique involves immobilizing a biological molecule such as a protein, DNA, etc., at a surface via a self-assembled monolayer, affecting the biological molecule via, for example, biological binding, inducing a change in conformation via a prion, etc., and detecting an electronic property change in the molecule via a change in impedance associated with an electronic circuit addressed by the biological molecule. This technique facilitates combinatorial array detection articles.





FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL AM AT AU AZ BA BB BE BF BG BJ BR BY CA CF CG CH CI CM CN CU CZ DE DK EE	Albania Armenia Austria Austria Austria Austria Azerbaijan Bosnia and Herzegovina Barbados Belgium Burkina Faso Bulgaria Benin Brazil Belarus Canada Central African Republic Congo Switzerland Côte d'Ivone Cameroon China Cuba Czech Republic Germany Denmark Estonia	ES FI FR GA GB GE GH GN GR HU IE IL IS IT JP KE KG KP KR KZ LC LI LK LR	Spain Finland France Cabon United Kingdom Georgia Ghana Guinea Greece Hungary Ireland Israel Iceland Italy Japan Kenya Kyrgy/stan Democratic People's Republic of Korea Republic of Korea Razakstan Saint Lucia Liceltenstein Sri Lanka Luberia	LS LT LU LV MC MD MG MK ML MN MR MW MX NE NL NO NZ PL PT RO SE SG	Lesotho Lithuania Luxembourg Latvia Monaco Republic of Moldova Madagascar The former Yugoslav Republic of Macedonia Mali Mongolia Mauritania Malawi Mexico Niger Netherlands Norway New Zealand Potand' Portugal Romania Russian Federation Sudan Sweden Singapore	SI SK SN SZ TD TG TJ TM TR TT UA UG US UZ VN YU ZW	Slovenia Slovakia Senegal Swaziland Chad Togo Tajikistan Turkuenistan Turkey Trinidad and Tobago Ukraine Uganda United States of America Uzbekistan Viet Nam Yugoslavia Zimbabwe	
--	---	--	---	---	--	--	--	--

WO 98/31839 PCT/US98/01144

ELECTRONIC-PROPERTY PROBING OF BIOLOGICAL MOLECULES AT SURFACES

Related Application

This application is a continuation-in-part of U.S. application serial no. 08/804,883 by Cynthia C. Bamdad entitled "Electronic-Property Probing of Biological Molecules at Surfaces", filed February 24, 1997, which is a continuation-in part of U.S. application serial no. 08/786,153 by Cynthia C. Bamdad entitled "Surface-Immobilized Nucleic Acid and Electron-Transfer Devices and Methods Employing the Same", filed January 21, 1997.

10

20

25

5

Field of the Invention

The present invention relates generally to the derivatization of surfaces for determination of analytes, for example from a fluid medium using a biological binding partner of an analyte, and more particularly to the formation, on a surface, of a self-assembled molecular monolayer including a biological species such as a nucleic acid strand, and the use of the self-assembled monolayer in a probe that acts to detect molecular recognition via electronic properties of the biological species.

Background of the Invention

Biochemical analyses are invaluable, routine tools in health-related fields such as immunology, pharmacology, gene therapy, and the like. In order to successfully implement therapeutic control of biological processes, it is imperative that an understanding of biological binding between various species is gained. Indeed, an understanding of biological binding between various species is important for many varied fields of science.

Many biochemical analytical methods involve immobilization of a biological binding partner of a biological molecule on a surface, exposure of the surface to a medium suspected of containing the molecule, and determination of the existence or extent of molecule coupling to the surface-immobilized binding partner.

The study of biological binding involving nucleic acid at surfaces has been hindered by the difficulty in immobilizing a single strand of nucleic acid at a surface without also immobilizing the complement of that strand. Where a single strand of nucleic acid is immobilized at a surface with its complement, it is not available for interaction by itself.

Electron transfer through model enzymes has been studied, and several theoretical models predict rates of transfer through these enzymes (Chidsey, C.E.D., "Free Energy and Temperature Dependence of Electron Transfer at the Metalelectrolyte Interface," Science 251 (1991), pp. 919-922). Comparison of predicted electron transfer rates with the time required for electrons to travel a finite distance within a protein has led to the conclusion that electrons traverse a pathway of chemical bonds such as covalent or hydrogen bonds (J. N. Onuchic, D.N. Beratan, J.R. Winkler, and H.B. Gray, Ann. Rev. Biophys. Biomol. Struct., 21 349 (1992); D.N. Baratan, J.N. Onuchic, J.R. Winkler and H.B. Gray, Science, 258 1740 (1992); J.J. Regan, S.M. Risser, D.N. Beratan, and J.N. Onuchic, J. Phys. Chem., 97 13083 (1993)), but do not travel through vacant space (S.M. Risser, D.N. Beratan, and T.J. Meade, J. Am. Chem. Soc., 115 2508 (1993)). This finding was later modified to include electron transfer between π -stacked electron systems (F. Barigelletti, L. Flamigni, V. Balzani, J.P. Collin, J.P. Sauvage, A. Sour, E.C. Constable, and A.C.M.W. Thompson, J. Am. Chem. Soc., 116 7692 (1994); J.N. Onuchic and D.N. Beratan, J. im. Chem. Soc., 109 6771 (1987)). Subsequently, several groups measured rates of electron transfer through electroactive proteins (enzymes) using modified or unmodified electrodes, then microelectrodes (Hill, H.A.O., Klein, N.P., Murthy, A.S.N., Psalti, I.S.N., Chemical Sensors and Instrumentation, (1989) pp. 105-113; Armstrong, F.A., Bond, A.M., Hill, H.A.O., Psalti, I.S.N., Zoski, C.G., J. Phys. Chem. 93, (1989) pp. 6485-6493).

One drawback in these studies is that direct adsorption of protein onto an electrode
typically resulted in loss of conductivity, presumably due to protein denaturation. A hydrophilic
molecule (promoter) therefore was adsorbed to an electrode prior to adsorption of the
electroactive protein in some cases. The promoter layer is designed to bind the protein of interest
through hydrogen bonds, giving the electrons a suitable pathway. Notably, in these studies,
electrons were observed to travel through an inert molecule, then through the electroactive
molecule (Hill, H.A.O. and Lawrence, G.A., "Some Consequences of Mixed and Dilute Surface
Modification of Gold Electrodes for Protein Electrochemistry," *J. Electroanal. Chem.* 270 (1989)
pp. 309-318). The amplitude of the signal was dependent upon the potential difference.

Rates of electron transfer have also been measured through DNA (C.J. Murphy, M.R. Arkin, Y. Jenkins, N.D. Ghatlia, S.H. Bossman, N.J. Turro and J.K. Barton, *Science*, **262** 1025 (1993)). It has been shown that the rate of electron transfer through double-stranded DNA is much faster than through single-stranded DNA (T.J. Meade and Jon F. Kayyem, *Angew. Chem. Int. Ed. Engl.*, **34** 3, pp. 352-354 (1995), "Electron Transfer through DNA: Site-Specific

Modification of Duplex DNA with Ruthenium Donors and Acceptors").

Co-pending, commonly-owned U.S. patent application serial no. 08/312,388, filed September 26, 1994 by Bamdad, et al., describes a technique for immobilization of single-stranded DNA at a surface as part of a self-assembled monolayer, and use of the arrangement in determination of biological binding partners of the DNA via Surface Plasmon Resonance (SPR), a technique that measures the very slight changes in mass that occurs at a surface upon biological binding of a binding partner to the surface-immobilized species.

Accordingly, it is an object of the invention to provide techniques for studying molecular interactions at surfaces.

10

15

Summary of the Invention

The foregoing and other objects and advantages of the invention are achieved by providing a molecule having a formula X-R-Ch, in which X represents a functional group that adheres to a surface such as a gold surface. R represents a spacer moiety that promotes formation of a self-assembled monolayer of a plurality of the molecules, and Ch represents a bidenti te. tridentate, or quadradentate chelating agent that coordinates a metal ion. The chelating agent includes a chelating moiety and a non-chelating linker moiety, such that it can be covalently linked via its linker moiety to the spacer moiety while allowing the chelating moiety to coordinate a metal ion. According to a preferred aspect of the invention a metal ion is coordinated to the chelating agent, and a binding partner of a target molecule is coordinated to the metal ion. This arrangement is facilitated by selecting the chelating agent in conjunction with the metal ion such that the chelating agent coordinates the metal ion without completely filling the ion's coordination sites, allowing the binding partner to coordinate the metal ion via coordination sites not filled by the chelating agent. According to one aspect of the invention the binding partner is a biological species that includes a polyamino acid tag, such as a tag made up of at least two histidine residues, that coordinates the metal ion. In this context the term "adhere" means to chemisorb in the manner in which, for example, alkyl thiols chemisorb to gold.

The present invention also provides a species having a formula X-R-Ch-M-BP-BMol, in which X represents a functional group that adheres to a surface, R represents self-assembled monolayer-promoting spacer moiety, Ch represents a chelating agent that coordinates a metal ion. M represents a metal ion coordinated by the chelating agent, BP represents a biological binding partner of a biological molecule, and BMol represents the biological molecule. The

binding partner is coordinated to the metal ion.

The invention also provides an article including a solid phase that has a surface. A plurality of chelating agents are immobilized at the surface in such a way that essentially each of the chelating agents is oriented so as that the chelating moiety of the agent, that is the electron donating portions of the agent, face in a direction away from the surface and is unencumbered by species, such as other chelating agents, that would interfere with the chelating function. This can be accomplished by isolating the chelating agent at the surface by non-chelating species. In this way each chelating agent can coordinate a metal ion so as to expose in a direction away from the surface at least two free metal coordination sites. According to one aspect of the invention the article includes a surface and a self-assembled mixed monolayer adhered to the surface and formed of at least a first and a second species. The first species has a formula X-R-Ch, where X, R, and Ch are each selected such that X represents a functional group that adheres to the surface. R represents a spacer moiety that promotes self-assembly of the mixed monolayer, and Ch represents a chelating agent that coordinates a metal ion. The second species is selected to form a mixed self-assembled monolayer with the first species, and the mixed monolayer is made up of at least 70 mol percent of the second species. The second species preferably is a species selected to inhibit non-specific binding of a protein to the surface.

According to a preferred aspect, the article is suitable for capturing a biological molecule. According to this aspect a self-assembled mixed monolayer, formed of a first species and a second species, is adhered to the surface. The first species has a formula X-R-Ch-M-BP, where X, R, Ch, M, and BP are each selected such that X represents a functional group that adheres to the surface, R represents a spacer moiety that promotes self-assembly of the mixed monolayer, Ch represents a chelating agent that coordinates a metal ion. M represents a metal ion, and BP represents a binding partner of the biological molecule. The binding partner is coordinated to the metal ion. The second species is selected to form a mixed, self-assembled monolayer with the first species, and according to a preferred aspect the second species has a formula, X-R-O-(CH₂CH₂-O)_n-H, in which X represents a functional group that adheres to the surface, R represents a spacer moiety that promotes formation of a self-assembled monolayer of a plurality of the molecules, and n is from one to ten. The article can be constructed and arranged to facilitate instrumental determination of an analyte, and according to a preferred aspect is a biosensor element such as a SPR chip.

The present invention also provides a method of making an article for capturing a target

20

WO 98/31839 - 5 -

molecule. The method of making the article includes formulating a solution containing a mixture of at least a first and a second species, and exposing to the solution a surface of the article for a period of time sufficient to form a self-assembled mixed monolayer of the first and second species on the surface. The first species has a formula X-R-Ch as described above. The second species is selected to form a mixed self-assembled monolayer with the first species, and the second and first species are present in the solution at a molar ratio of at least 70:30.

The present invention also provides a method of capturing a biological molecule. The method involves contacting a medium suspected of containing the biological molecule with a solid phase that has a surface carrying a plurality of binding partners of the biological molecule, in which essentially all of the binding partners are oriented to expose away from the surface a recognition region for the biological molecule. The biological molecule then is allowed to biologically bind to the binding partner, and the biological molecule bound to the binding partner then can be determined. According to one aspect the method involves providing a solid phase having a surface, a chelating agent immobilized at the surface, a metal ion coordinated by the chelating agent, and a biological binding partner of the biological molecule coordinated to the metal ion. According to this aspect the surface is brought into contact with a medium suspected of containing the biological molecule for a period of time sufficient to allow the biological molecule to biologically bind to the binding partner.

The present invention provides another method of capturing a biological molecule. The method involves providing a solid phase having a surface, and a metal ion immobilized at the surface in such a way that the metal ion has at least two free coordination sites. A biological binding partner of a biological molecule is coordinated to the metal ion via a polyamino acid tag, and a medium containing the biological molecule is brought into contact with the surface, whereupon the biological molecule is allowed to biologically bind to the binding partner. The biological molecule then can be determined.

This method involves providing a solid phase that has a surface having adhered thereto a species having a formula X-R-Ch-M-BP, in which X represents a functional group that adheres to the surface, R represents a self-assembled monolayer-promoting spacer moiety, Ch represents a chelating agent that coordinates a metal ion, M represents a metal ion coordinated by the chelating agent, and BP represents a binding partner of the biological molecule, coordinated to the metal ion. A target molecule then is allowed to biologically bind to the binding partner. The

WO 98/31839 - 6 - PCT/US98/01144

biological molecule then can be determined, for example by detecting a physical change associated with the surface.

An article provided in accordance with the invention can be a biosensor element, such as a SPR chip, and the determination carried out by measuring surface plasmon resonance associated with the chip. The methods of the invention that involve capturing a molecule can involve removal of a preselected molecule, such as a biological molecule, from a fluid medium.

The present invention also provides sensing elements fashioned as described above and suitable for use in a biosensor, for determination of a biological molecule and in particular a molecule that is a binding partner of a nucleic acid strand. A particularly preferred sensing element includes a substrate, a metal film having a surface, and a self-assembled monolayer of a species X-R-NA or X-R-NA-NAB. X represents a functional group that adheres to the surface, R represents a spacer moiety that promotes formation of a self-assembled monolayer of a plurality of the species, NA represents a nucleic acid strand, and NAB represents a nucleic acid strand that is a binding partner of NA and a binding partner of the biological molecule to be determined.

The present invention also provides a kit including an article having a surface and a molecule X-R-Ch, both as described above. The kit can include M and BP, either separately or combined as species X-R-Ch-M or X-R-Ch-M-BP, where X, R, Ch, M, and BP are as described herein. The kit also can include X-R-NA, optionally with NAB, or X-R-NA-NAB as described herein.

Another aspect of the invention is the article formed when the foregoing molecule(s) is adhered to a surface, preferably gold. In this embodiment the article has a chelating agent as described above attached to a spacer moiety as described above which in turn is adhered via X.

In another aspect the invention provides a self-assembled monolayer including a species X-R-Ch as described above, wherein at least 90% of the Ch units are isolated from all other Ch units. In one embodiment, the Ch units are isolated from each other by at least 5 nm. They can be isolated from each other by a biologically-inert self-assembled monolayer-forming species.

In another aspect, the invention provides a self-assembled monolayer-forming species including a nucleic acid strand. The nucleic acid strand can be single-stranded DNA or double-stranded DNA, or another species. The nucleic acid strand can be a single nucleic acid strand free of hybridization from a complementary strand, and/or can form a part of a self-assembled monolayer of other nucleic acid strand species. The nucleic acid strand can be covalently

15

coupled to a self-assembled monolayer-forming species, thereby forming a part of a self-assembled monolayer.

The invention also provides a single nucleic acid strand that is immobilized at a surface, which immobilization can be covalent immobilization, and the strand is not removable from the surface under disassociation conditions and is free of hybridization to any nucleic acid strand not removable from the surface under disassociation conditions. The nucleic acid strand, according to this aspect, can be hybridized to a complementary nucleic acid strand that is disassociable from the single strand under disassociation conditions.

According to another aspect, the invention provides a surface on which is a self-assembled monolayer including a plurality of self-assembled monolayer-forming species each including a nucleic acid strand. At least 90% of the nucleic acid strands are biologically isolated from all other nucleic acid strands in this aspect. At least 90% of the nucleic acid strands are isolated from each other by at least 5 nm according to one embodiment, and can be isolated from each other by a biologically inert self-assembled monolayer-forming species.

According to another aspect, the invention provides a method including providing a single nucleic acid strand immobilized at a surface, and allowing a biological binding partner of the nucleic acid strand to biologically bind to the strand. The single nucleic acid strand can be covalently immobilized to the surface or immobilized in any other way as part of a self-assembled monolayer in preferred embodiments, and preferably is isolated from other single nucleic acid strands as described above. Alternatively, double-stranded nucleic acid can be immobilized at the surface in this way. In one embodiment, the biological binding partner is a nucleic acid strand that is complementary to the nucleic acid strand immobilized at the surface. In another embodiment the binding partner is a protein or the like.

According to another aspect the invention provides a method including introducing an electron into a first biological species. The first biological species is one of a pair of biological binding partners defined by the first biological species and a second biological species. The electron then is passed through the first species into the second species, and removed from the second species. The first and second species can be nucleic acid strands that are complimentary, and one or both can be immobilized at a surface of a respective article. When the respective articles are electrodes included in an electrical circuit, the method can involve determining electronic transfer through the biological species.

An array of biological species of this type can be provided on a surface. That is,

15

20

25

:

WO 98/31839 - 8 -

biologically distinct species (species including binding properties distinct from each other; species that will not biologically bind to each other) can be immobilized on the surface, preferably via a self-assembled monolayer, preferably covalently, and preferably in a manner in which they are isolated from each other by at least 5 nanometers. These distinct biological species can be addressed by other biological species that might bind biologically to one or more of these species, and where binding occurs electrical current flows, and identification of a biological species is made.

In another aspect, the invention provides a method that involves passing an electron through a biological species linked to a self-assembled monolayer-forming species immobilized at a surface of an article.

In another aspect, the invention involves a method including providing a first biological species that is linked to a surface of a first article via a self-assembled monolayer. A second biological species also is linked to the same surface via a self-assembled monolayer. An electron is passed through the first biological species while the second biological species does not pass an electron. The electron passed through the first biological species can be passed from the article into the species, or from the species into the article.

According to another aspect the invention provides a method including providing a first biological species immobilized at a surface of a first article and a second biological species immobilized to the surface of the first article. An electron is passed through the first biological species while the second biological species does not pass an electron.

The invention also provides an article including a first electrode having a surface, a second electrode having a surface, and an electrical circuit connecting the first and second electrodes. A first biological species is immobilized at a surface of the first electrode, and a biological binding partner of the first biological species is immobilized at the second electrode.

In another aspect the invention provides a method involving determining the interaction of a first molecule with a second molecule by detecting a change in electronic configuration of the second molecule due to interaction of the second molecule with the first molecule. In one aspect the determining step involves exposing the second molecule to a medium suspected of containing the first molecule and determining the presence of the first molecule in the medium. The second molecule can be immobilized at a surface via a spacer moiety and the determining step can involve the first molecule to interact with the second molecule and determining a change in electronic configuration in the second molecule via detection involving electron transfer

10

20

WO 98/31839 - 9 - PCT/US98/01144

through the spacer moiety between the surface and the second molecule. The determining step can also involve determining a change in impedance of a system including the second molecule, after interaction with the first molecule, relative to an impedance measurement of the system without interaction with the first molecule.

The invention also provides a method involving immobilizing a protein at a surface, inducing a change in the conformation of the protein at the surface, and detecting the change in conformation.

The invention also provides a method involving providing a protein exposing the protein to a prion or associated cofactor, and determining a change in conformation of the protein induced by exposure of the protein to the prion.

Also provided is a method involving determining a disease state involving mis-folded protein in a physiological sample of a living mammal

Other advantages, novel features, and objects of the invention will become apparent from the following detailed description of the invention when considered in conjunction with the accompanying drawings, which are schematic and which are not intended to be drawn to scale. In the figures, each identical or nearly identical component that is illustrated in various figures is represented by a single numeral. For purposes of clarity, not every component is labeled in every figure.

20 Brief Description of the Drawings

Fig. 1 is a SPR sensorgram illustrating response of a prior art chip carrying Gal 11 to SRB2:

Fig. 2 is a SPR sensorgram illustrating response of a chip derivatized in accordance with the present invention, and carrying Gal 11, to SRB2;

Fig. 3 is a SPR sensorgram illustrating response of a prior art chip carrying a T cell receptor (ABC) to monoclonal antibodies C1 and BF1;

Fig. 4 is a SPR sensorgram illustrating response of a chip, derivatized in accordance with the present invention and carrying a T cell receptor (ABC), to monoclonal antibodies C1 and BF1; and

Fig. 5 is a SPR sensorgram illustrating response of a chip, derivatized in accordance with the invention to present a single-stranded nucleotide sequence, first to double-stranded DNA carrying a single-stranded "tail" scrambled with respect to that presented by the chip, and then to

25

30



a single-stranded "tail" complementary to that presented by the chip;

Fig. 6 is a SPR sensorgram illustrating response of the chip of Fig. 5 to double-stranded DNA without a "tail";

- Fig. 7 is a SPR sensorgram illustrating response of a chip carrying double-stranded DNA including 2Gal4 binding sites to Gal4 and to LexB17;
 - Fig. 8 is a schematic illustration of a surface derivatized with a mixed SAM formed of an inert major component and a minor component that includes a nucleic acid strand;
 - Fig. 9 is a schematic illustration of the SAM of Fig. 8 following hybridization with double-stranded DNA having a "tail" complementary to the nucleic acid strand presented by the surface after hybridization, ligation, and restriction;
 - Fig. 10 is a schematic illustration of hybridization of double-stranded DNA, ligated with the same restriction enzyme used to ligate the double-stranded DNA immobilized at the surface, to the DNA of the surface followed by ligation and disassociation;
- Fig. 11 is a schematic representation of nucleic acid strands 72 as part of a SAM on an electrode 66 and complementary nucleic acid strands 70 as part of a SAM on electrode 62 with an electrical circuit including the electrodes;
 - Fig. 12 is a schematic illustration of the arrangement of Fig. 11 in which complementary nucleic acid strands 70 and 72 are hybridized, completing an electrical circuit; and
 - Fig. 13 is a schematic illustration of an array of different biological species immobilized at one electrode, and an array of similar biological species immobilized at another electrode, for use in electron transfer probe analysis;
 - Fig. 14 is a schematic illustration of a single-electrode electronic property probe for biological molecules;
 - Fig. 15 is a dual-electrode electronic property probe for biological molecules;
 - Fig. 16 is a schematic illustration of another dual-electrode electronic property probe for biological molecules;
 - Fig. 17 is a schematic representation of yet another system for determining a change in an electronic property of a surface-immobilized molecule for drug screening; and
 - Fig. 18 is a schematic representation of a cyclic backbone structure providing covalent surface immobilization for attached molecules as part of a SAM.

Detailed Description of the Invention

Co-pending, commonly-owned application serial no. 08/312,388 entitled "Molecular Recognition at Surfaces Derivatized with Self-Assembled Monolayers" by Bamdad, et al., filed September 26, 1994; co-pending, commonly-owned application serial no. 08/786,187 entitled "Molecular Recognition at Surfaces Derivatized with Self-Assembled Monolayers" by Bamdad, et al., filed January 21, 1997, co-pending, commonly-owned application serial no. 08/786,153 entitled "Surface-Immobilized Nucleic Acid and Electron-Transfer Devices and Methods Employing the Same" by Bamdad, et al., filed January 21, 1997, and co-pending, commonly-owned application serial no. 08/616,929, filed March 15, 1996 by Kim, et al., entitled "Method of Forming Articles and Patterning Surfaces via Capillary Micromolding"; U.S. application serial no. 08/131,838. filed October 4, 1993, entitled "Method of Formation of Microstamped Patterns on Plates for Adhesion of Cells and Other Biological Materials, Devices and Uses Therefor", by Singhvi, et al.; and U.S. patent no. 5,512,131, issued April 30, 1996 to Kumar, et al., all are incorporated herein by reference.

15 Nomenclature

20

The following definitions are provided to facilitate a clear understanding of the present invention.

The term, "chelating agent" refers to an organic molecule having unshared electron pairs available for donation to a metal ion. The metal ion is in this way coordinated by the chelating agent. Two or more neighboring amino acids can act as a chelating agent.

The terms, "bidentate chelating agent", "tridentate chelating agent", and "quadradentate chelating agent" refer to chelating agents having, respectively, two, three, and four electron pairs readily available for simultaneous donation to a metal ion coordinated by the chelating agent.

The term "biological binding" refers to the interaction between a corresponding pair of molecules that exhibit mutual affinity or binding capacity, typically specific or non-specific binding or interaction, including biochemical, physiological, and/or pharmaceutical interactions. Biological binding defines a type of interaction that occurs between pairs of molecules including proteins, nucleic acids, glycoproteins, carbohydrates, hormones and the like. Specific examples include antibody/antigen, antibody/hapten, enzyme/substrate, enzyme/inhibitor,

enzyme/cofactor, binding protein/substrate, carrier protein/substrate, lectin/carbohydrate, receptor/hormone, receptor/effector, complementary strands of nucleic acid, protein/nucleic acid repressor/inducer, ligand/cell surface receptor, virus/ligand, etc.

The term "binding partner" refers to a molecule that can undergo biological binding with a particular biological molecule. For example, Protein A is a binding partner of the biological molecule IgG, and vice versa.

The term "biological molecule" refers to a molecule that can undergo biological binding with a particular biological binding partner.

The term "recognition region" refers to an area of a binding partner that recognizes a corresponding biological molecule and that facilitates biological binding with the molecule, and also refers to the corresponding region on the biological molecule. Recognition regions are typified by sequences of amino acids, molecular domains that promote van der Waals interactions, areas of corresponding molecules that interact physically as a molecular "lock and key", and the like.

The term "biologically inert", when describing the relationship between two species means that the two species do not undergo biological binding.

The term "coordination site" refers to a point on a metal ion that can accept an electron pair donated, for example, by a chelating agent.

The term "free coordination site" refers to a coordination site on a metal ion that is occupied by a water molecule or other species that is weakly donating relative to a polyamino acid tag, such as a histidine tag.

The term "coordination number" refers to the number of coordination sites on a metal ion that are available for accepting an electron pair.

The term "coordinate bond" refers to an interaction between an electron pair donor and a coordination site on a metal ion leading to an attractive force between the electron pair donor and the metal ion.

The term "coordination" refers to an interaction in which one multi-electron pair donor, such as a chelating agent or a polyamino acid tag acting as a chelating agent, coordinatively bonds (is "coordinated") to one metal ion with a degree of stability great enough that an interaction that relies upon such coordination for detection can be determined by a biosensor. The metal ion is coordinated by the multi-electron pair donor.

The term "solid phase" refers to any material insoluble in a medium containing a target molecule or biological molecule that is desirably captured in accordance with the invention. This term can refer to a metal film, optionally provided on a substrate.

The term "surface" refers to the outermost accessible molecular domain of a solid phase.

15

WO 98/31839 - 13 -

The term "capturing" refers to the analysis, recovery, detection, or other qualitative or quantitative determination of an analyte in a particular medium. The medium is generally fluid, typically aqueous. The term, "captured", refers to a state of being removed from a medium onto a surface.

The term "target molecule" refers to a molecule, present in a medium, which is the object of attempted capture.

The term "determining" refers to quantitative or qualitative analysis of a species via, for example, spectroscopy, ellipsometry, piezoelectric measurement, immunoassay, and the like.

The term "immobilized", used with respect to a species, refers to a condition in which the species is attached to a surface with an attractive force stronger than attractive forces that are present in the intended environment of use of the surface and that act on the species, for example solvating and turbulent forces. Coordinate and covalent bonds are representative of attractive forces stronger than typical environmental forces. For example, a chelating agent immobilized at a surface, the surface being used to capture a biological molecule from a fluid medium, is attracted to the surface with a force stronger than forces acting on the chelating agent in the fluid medium, for example solvating and turbulent forces.

The term "non-specific binding" (NSB) refers to interaction between any species, present in a medium from which a target or biological molecule is desirably captured, and a binding partner or other species immobilized at a surface, other than desired biological binding between the biological molecule and the binding partner.

The term "self-assembled monolayer" refers to a relatively ordered assembly of molecules spontaneously chemisorbed on a surface, in which the molecules are oriented approximately parallel to each other and roughly perpendicular to the surface. Each of the molecules includes a functional group that adheres to the surface, and a portion that interacts with neighboring molecules in the monolayer to form the relatively ordered array. See Laibinis, P. E.; Hickman, J.; Wrighton, M. S.; Whitesides, G. M. Science 245, 845 (1989), Bain, C.; Evall, J.; Whitesides, G. M. J. Am. Chem. Soc. 111, 7155-7164 (1989), Bain, C.; Whitesides, G. M. J. Am. Chem. Soc. 111, 7164-7175 (1989), each of which is incorporated herein by reference.

The term "self-assembled mixed monolayer" refers to a heterogeneous self-assembled monolayer, that is, one made up of a relatively ordered assembly of at least two different molecules.

The present invention provides a technique for molecular recognition at surfaces that

5

10

WO 98/31839 - 14 - PCT/US98/01144

involves electron transfer through a biological species immobilized at the surface. The surface presenting the immobilized biological species is exposed to a biological binding partner of the biological species, or a biological binding partner of a biological binding partner of the species, and electron transfer through the biological species and the biological binding partner determined.

In the case of many biological molecules, biological binding is extremely sensitive to orientation and conformation of the members involved in the binding, that is, the biological molecule and the binding partner. Accordingly, the manner of presentation of the binding partner at the surface to a medium containing the biological molecule is directly related to the sensitivity, and success, of the technique. The present invention presents a biological species such as a nucleic acid strand at the surface in a manner in which biological binding with the nucleic acid strand at the surface is facilitated (as described in the co-pending, commonly-owned applications both entitled "Molecular Recognition at Surfaces Derivatized with Self-Assembled Monolayers", one filed September 26, 1994 and assigned serial no. 08/312,388, and the other filed January 21, 1997 and assigned serial no. 08/786,187) and makes use of this presentation in a unique analysis technique.

As discussed in the above-referenced co-pending applications, a self-assembled monolayer (SAM) including a species defining in part a nucleic acid strand (or other biologically-binding species, a nucleic acid strand exemplifying such species here), is made up of a mixture of a nucleic acid strand SAM-forming species and a species that is inert with respect to binding in which the nucleic acid strand is involved. Such a SAM can be formed on a surface by mixing the nucleic acid strand species and the inert species in a solvent and exposing the surface to the solvent. The relative amount of nucleic acid strand SAM-forming species in the solvent (and in the resultant SAM; the species' ratio in the solvent approximates or equals the species' ratio in the SAM) affects the average distance between individual nucleic acid strands presented at the surface; a higher ratio of inert species to nucleic acid strand species results in greater distance between nucleic acid strands in the SAM on the surface. It is generally advantageous to separate the individual nucleic acid strands from each other to an extent great enough to prevent inter-strand interaction, and interaction between other biological species coupled directly or indirectly to each strand, and sensitivity of a particular technique can be maximized by minimizing inter-strand distance on the surface (maximizing nucleic acid concentration). Generally, the molar ratio of the inert species to the nucleic acid species in the SAM is at least

5

WO 98/31839 - 15 - PCT/US98/01144

SAM is at least 70:30. According to a preferred embodiment, the molar ratio is at least 80:20, more preferably at least 90:10, and more preferably still at least about 95:5. In some cases, a molar ratio of inert species to nucleic acid species of about 99.5:0.5 is useful. A variety of spectroscopic techniques are available in the art for determining a final ratio of inert and nucleic acid species immobilized at a surface.

As mentioned, the major component in the SAM preferably is selected to expose, to the medium containing a target biological molecule, a functionality that inhibits NSB. Specifically, a functionality is selected to inhibit NSB of species present at relatively high concentration in a medium in which the biological molecule is presented to the surface. That is, the second species is selected among those that include chemical functionality, at the end of the molecule opposite the functionality that adheres to the surface, that does not bind species in the medium (the end opposite the functionality that adheres to the surface is presented to the medium, or "exposed" at the surface).

The second species has a formula X-R-NSBi, where NSBi is a NSB-inhibitor. NSBi can be selected from such groups as -CH₃; -OI1; -O(CH₂)_nH, where n = 1-15, preferably 1-7; -CONH(CH₂)_nH, where n = 1-15, preferably 1-7; -NIICO(CH₂)_nH, where n = 1-15, preferably 1-7; -COOH; -O(CH₂CH₂-O)_nH (where n = 1-10); -(CF₂)_nCF₃, where n = 1-20, preferably 1-8; olefins, and the like. Preferred are -CH₃, -OH, and -O(CH₂CH₂-O)_nII. According to a preferred embodiment in which a medium containing biological, particularly proteinaceous, species contacts the surface, the second species preferably has a formula X-R-O-(CH₂CH₂-O)_n-II, in which X represents a functional group that adheres to the surface. R represents a spacer moiety that promotes formation of a self-assembled monolayer of a plurality of the molecules, and n is from one to ten. According to a more preferred embodiment, n is from 2 to 5, and according to a particularly preferred embodiment, n is 3.

The surface, and the functional group X that adheres to the surface, can be selected among a wide variety known to those of skill in the field of surface science. A nonlimiting exemplary list of combinations of surface materials and functional groups X suitable for use in the present invention follows. Although the following list categorizes certain preferred materials with certain preferred functional groups which firmly bind thereto, many of the following functional groups would be suitable for use with exemplary materials with which they are not categorized, and any and all such combinations are within the scope of the present invention. Preferred surface materials include metals such as gold, silver, copper, cadmium, zinc,

15

20

palladium, platinum, mercury, lead, iron, chromium, manganese, tungsten, and any alloys of the above with sulfer-containing functional groups X such as thiols, sulfides, disulfides, and the like; doped or undoped silicon with silanes and chlorosilanes; metal oxides such as silica, alumina, quartz, glass, and the like with carboxylic acids; platinum and palladium with nitriles and isonitriles; and copper with hydroxamic acids. Additional suitable functional groups include acid chlorides, anhydrides, sulfonyl groups, phosphoryl groups, hydroxyl groups and amino acid groups. Additional surface materials include germanium, gallium, arsenic, and gallium arsenide. Additionally, epoxy compounds, polysulfone compounds, plastics and other polymers may find use as a surface material in the present invention. Additional materials and functional groups suitable for use in the present invention can be found in U.S. Patent No. 5,079,600, issued January 7, 1992, incorporated herein by reference.

According to a more preferred embodiment, a combination of gold as surface material and a functional group X having at least one sulfer-containing functional group such as a thiol, sulfide, or disulfide is selected. The interaction between gold and such sulfer-containing functional groups is a well-studied science, and a nonlimiting representative exemplary list of such sulfer-containing functionalities may be found in an article entitled "Wet Chemical Approaches to the Characterization of Organic Surfaces: Self-Assembled Monolayers, Wetting and the Physical-Organic Chemistry of the Solid-Liquid Interface", by G.W. Whitesides and Paul E. Laibinis, Langmuir, 6, 87 (1990), incorporated herein by reference. Particularly preferred in the present invention is a gold surface, and a thiol as functional group X.

The spacer moiety R can be selected from among organic spacer moieties that promote formation of self-assembled monolayers. Such moieties are well-known to those of ordinary skill in the art, as described in the above-referenced articles by Laibinis, et al. Science 245, 845 (1989), Bain, et al. J. Am. Chem. Soc. 111, 7155-7164 (1989), and Bain, C, et al. J. Am. Chem. Soc. 111, 7164-7175 (1989). Preferred moieties R are hydrocarbon chains optionally interrupted by hetero groups, of a length of at least eight carbon atoms. As used herein, "hydrocarbon" is meant to define includes alkyl, alkenyl, alkynyl, cycloalkyl, aryl, alkaryl, aralkyl, and the like. Hetero groups can include -O-, -CONH-, -CONHCO-, -NH-, -CSNH-, -CO-, -CS-, -S-, -SO-, -(OCH₂CH₂)_nR (where n = 1-20, preferably 1-8), -(CF₂)_n- (where n = 1-20, preferably 1-8), olefins, and the like. It is important that the R is a self-assembled monolayer-promoting moiety. Whether or not a particular moiety promotes formation of a self-assembled monolayer can be routinely determined by one of ordinary skill in the art, optionally in accordance with the

20

teachings of the preceding references, using for example surface spectroscopic techniques.

According to a preferred embodiment, $R = -(CH_2)_n$ -, where n is from about 8 to about 24, preferably from about 10 to about 20, most preferably from about 9 to about 16. According to an embodiment of the invention in which R is a moiety in the group X-R-Ch, R preferably comprises $-(CH_2)_n$ -O(CH_2CH_2 -O)_m-, where n = 4-20, preferably 8-14, and m = 1-10, preferably 2-5. a variety of moieties R can be used on different molecules forming a self-assembled mixed monolayer, so long as other requirements described herein are met.

R should also be selected to be chemically stable to reagents used in the synthesis of a species into which it is incorporated. For example, if the species is formed by cleavage of a disulfide, R should be stable to reagents such as mercaptoethanol and dithiothreitol.

The metal ion is preferably selected from those that have at least four coordination sites, preferably six coordination sites, a non-limiting list of metal ions suitable includes Co^3 , $Cr^{3^{\circ}}$, $Hg^{2^{\circ}}$, $Pd^{2^{\circ}}$, $Pt^{2^{\circ}}$, $Pd^{4^{\circ}}$, $Pt^{4^{\circ}}$, $Rh^{3^{\circ}}$, $Ir^{3^{\circ}}$, $Ru^{3^{\circ}}$, $Co^{2^{\circ}}$, $Ni^{2^{\circ}}$, $Cu^{2^{\circ}}$, $Zn^{2^{\circ}}$, $Cd^{2^{\circ}}$, $Pb^{2^{\circ}}$, $Mn^{2^{\circ}}$, $Fe^{3^{\circ}}$, $Fe^{2^{\circ}}$, $Au^{3^{\circ}}$, Au^{4} , Ag° , Cu^{4} , $MO_2^{2^{\circ}}$, $Tl^{3^{\circ}}$, $Tl^{5^{\circ}}$, $Bi^{3^{\circ}}$, $CH_3Hg^{4^{\circ}}$, $Al^{3^{\circ}}$, $Ce^{3^{\circ}}$, $UO_2^{2^{\circ}}$, and $La^{3^{\circ}}$.

The chelating agent is preferably selected from bidentate, tridentate, and quadradentate chelating agents, and is selected in conjunction with the metal ion so that when the chelating agent coordinates the metal ion, at least two free coordination sites of the metal remain. The chelating agent and metal are selected so that the chelating agent can coordinate the metal ion with a degree of stability great enough that the metal ion will remain immobilized at the surface by the chelating agent.

Additionally, the chelating agent is selected as one that has a chelating moiety and a non-chelating linker moiety, such that it can be covalently linked via its linker moiety to the spacer moiety R while leaving the chelating moiety undisturbed by the covalent linkage and free to coordinate a metal ion. Alternatively, the chelating agent can be selected as one that can be modified via routine organic synthesis to include a non-chelating linker moiety, if such synthesis leaves undisturbed the chelating moiety. One of ordinary skill in the art will appreciate that the non-chelating linker moiety should provide functionality suitable for chemical linkage such as, for example, an amine, alcohol, carbamate, carboxylic acid, thiol, aldehyde, olefin, etc., for formation of an ester linkage, formation of an amide linkage, thiol displacement and this ether formation, and the like.

With the above considerations in mind, suitable chelating agents and corresponding metal ions can be selected by those of ordinary skill in the art. In accordance with such selection

15

20

25

reference can be made to "Chelating Agents and Metal Chelates", Dwyer, F. P.; Mellor, D. P., Academic Press, and "Critical Stability Constants", Martell, a. E.; Smith, R. M., Plenum Press, New York. These works describe a variety of chelating agents, and discuss the stability of coordination between chelating agents and metal ions. Preferably, a chelating agent and metal ion is selected such that the disassociation constant of the combination in aqueous solution is better than 10 nM at physiological pH, that is, such that at least one half of the metal ions are coordinated by chelating agent at a concentration of 10 nM.

A non-limiting exemplary list of suitable chelating agents includes nitrilotriacetic acid, 2,2'-bis(salicylideneamino)-6,6'-demethyldiphenyl, and 1,8-bis(a-pyridyl)-3,6-dithiaoctane.

In some cases it may be advantageous to test a particular chelating agent/metal ion pair to determine whether coordination will be sufficiently stable for use in the present invention. It is within the realm of routine experimentation to one of ordinary skill in the art to follow the teachings herein to immobilize a chelating agent at a surface, such as at a gold SPR chip surface, and then to test the interaction between the chelating agent and the metal ion via, for example, SPR spectroscopy under various conditions. In addition, preliminary screening can be carried out by reacting a prospective chelating agent and metal ion in solution and analyzing the solution spectroscopically. In this regard, reference can be made to, "Spectroscopy and Structure of Metal Chelate Compounds", Nakamoto, K.; McCarthy, S. J., Wiley, New York.

According to one aspect of the invention, an article suitable for capturing a biological molecule is provided. The article includes a self-assembled mixed monolayer of a first species and the second species as described above. The first species has a formula X-R-Ch-M-BP, where X, R, Ch, and M are as described above, and BP is a binding partner of a biological molecule, coordinated to the metal ion.

According to a preferred embodiment the binding partner includes a recognition region for the biological molecule, and a polyamino acid tag that can coordinate the metal ion and that is remote from the recognition region. a polyamino acid tag is meant to define a series of amino acids in proximity such that they can coordinate the at least two free coordination sites of the metal ion. According to a one embodiment, from 2 to about 10 neighboring amino acids such as, for example, neighboring histidines, lysines, arganines, glutamines, or any combination of these can serve as a polyamino acid tag. According to a preferred embodiment, the polyamino acid tag includes at least two, and more preferably from two to 10 neighboring histidines, and according to a particularly preferred embodiment the polyamino acid tag includes from about 3 to about 8

10

20

neighboring histidines. With reference to the above-identified work entitled, "Critical Stability Constants" (Martell, et al), selection of these and additional amino acids can be made in conjunction with selection of a metal ion M.

A variety of vectors that express proteins or fragments thereof containing a histidine tag are commercially available from, for example, Novagen, of Madison, WI. However, these vectors are designed to code proteins or fragments for metal chelate affinity chromatography. For purposes of metal chelate affinity chromatography, it is not important where on the vector the histidine tag lies. The purpose of the histidine tag in that application is solely to allow the chromatography solid phase to adsorb the protein. Therefore, it is not a priority in the commercialization of histidine-tagged proteins that the tag be placed at a location remote from the recognition region.

Where the binding partner is a polyamino acid, a polyamino acid tag can be expressed at a desired location (remote from the recognition region) in a number of ways known to those of ordinary skill in the art, for example by employing the polymerase chain reaction (PCR) to incorporate a nucleic acid sequence that encodes the polyamino acid tag at the appropriate position. Placement of a polyamino acid tag at a desired location is discussed more fully below.

Modification of a protein or fragment thereof by applying to the protein or fragment a polyamino acid tag at a location remote from the recognition region of the protein or fragment can be accomplished readily by one of ordinary skill in the art using, for example, recombinant technology. According to one method for such modification, a desired protein is grown from DNA that codes for the protein, and an expression vector. The protein is isolated and truncated at various amino acid positions, and the protein's specific active sequence (recognition region) elucidated by randomly mutating the resultant sequences. Alternatively, via sequence homology, a variety of similar proteins that recognize slightly different species are identified and their amino acid sequences determined. The various sequences are compared using a computer, and regions that are variable between the various proteins identified as recognition regions.

Subsequently a strand of DNA for a the desired protein fragment that is large enough to correctly fold can be sequenced with a polyamino acid tag at a location remote from the recognition region. DNA sequencing is routine in the art via. for example, PCR. With an expression vector, the desired polyamino acid-tagged protein fragment then can be readily grown and isolated.

The desired fragment will coordinate to the metal ion via the polyamino acid tag, and the

recognition region, remote from the tag, will not face the surface, but will be exposed to the medium containing or suspected of containing the binding partner of the protein fragment. As used herein, the term "remote" is meant to define a situation in which the polyamino acid tag is separated from the recognition region by a distance of at least about 20 amino acids, preferably at least about 40 amino acids.

The polyamino acid tagging technique of the invention can be applied to a variety of polyamino acids such as proteins, antibodies, antigens, polymers, and ligands.

Where the binding partner is a not a polyamino acid, it can be coupled chemically, for example covalently coupled, to a polyamino acid including a polyamino acid tag. In this case the coupling of the polyamino acid is effected on the binding partner at a location remote from the recognition region, and/or the polyamino acid that is coupled to the binding partner has a tag at a location remote from the location of coupling to the binding partner. Synthesis of such a species would be routine to those of ordinary skill in the art.

The present invention also provides a SAM-forming species that includes, at least in part, a nucleic acid strand, and can include double-stranded nucleic acid. The SAM-forming species according to this aspect of the invention when defining, in part, a SAM on a surface of an article, can define a sensing element suitable for use in a biosensor, and especially for use in determining a binding partner of a nucleic acid strand. Accordingly, the invention provides, in one embodiment, a species X-R-NA, or X-R-NA-NAB, as defined below. In another embodiment the invention provides a sensing element including a substrate and a self-assembled monolayer of a species X-R-NA, or X-R-NA-NAB, adhered to the surface of the substrate. The SAM contains, in preferred embodiments, one of these species in combination with an inert, nonbinding thiol as discussed above in combination with the species X-R-Ch, etc. The substrate preferably includes a metal surface, such as can be provided by a film of metal on a non-metal substrate. In this set of embodiments, X represents a functional group that adheres to the surface, R represents a spacer moiety that promotes formation of a self-assembled monolayer of a plurality of the species, NA represents a nucleic acid strand, and NAB represents a nucleic acid strand that is a binding partner of NA. NA or NAB can be a binding partner of a biological molecule to be determined.

Referring now to Figs. 8-10, SAMs including the species X-R-NA and X-R-NA-NAB are illustrated schematically. A description of this aspect of the invention is provided in greater detail below in Examples 10-14, and a brief description is provided here.

30

10

15

Referring to Fig. 8, an article 20 is illustrated which can define an SPR chip in one embodiment. Article 20 includes a substrate 22 having a surface 24 upon which is provided a SAM. In the embodiment illustrated, substrate 22 includes a film 26 on a surface thereof, the exposed surface of film 26 defining surface 24 of the overall substrate arrangement. Where an SPR chip is provided, substrates 22 can be glass and film 26 can be a thin gold film. A SAM 28 is provided on surface 24 of the substrate and includes a major component species 30 and a minor component species 32 which is a SAM-forming species including a nucleic acid strand 34. Nucleic acid strand 34 is preferably covalently coupled to a self-assembled monolayer-forming species X-R which forms a self-assembled monolayer with minor component 30. Minor component 30 is selected to have the ability to form an SAM with nucleic acid strand SAMforming species 32, is preferably of a length short enough, relative to species 32, that nucleic acid strand 34 of species 32 is exposed for hybridization, and otherwise can include a chemical functionality, exposed away from surface 24, that is desirable for whatever purpose article 20 serves. Typically, minor component 30 will netude an NSB-minimizing species such as a species terminating in polyethylene glycol. Synthesis of species 30 and species 32 is described below in the examples. The mixed monolayer including species 30 and species 32 includes nucleic acid strands 34 that are biologically isolated from all other nucleic acid strands. In particular, at least 90% of nucleic acid strands 34 are biologically isolated from other nucleic acid strands. As used herein, the term "biologically isolated" is meant to define a situation in which, were the nucleic acid strands complementary to each other, they would hybridize or 20 interact in another way. "Biologically-isolated" is also meant to define a situation in which if one nucleic acid strand included a region specific for a protein, and a protein were immobilized at that strand, if a neighboring nucleic acid strand had a sequence for that protein, the protein would not interact biologically (via biological binding or other recognition) with that strand. Specifically, at least 90% of nucleic acid strands 34 are isolated from all other nucleic acid strands, preferably by inert SAM-forming species 30, by at least 5 nm.

Article 20 can be used for a variety of purposes in which it is advantageous to expose a single-stranded nucleotide at a surface. In one preferred embodiment, with reference to Fig. 9, double-stranded nucleic acid (e.g. double-stranded DNA, dsDNA, as referred to hereinafter) 36, one of the strands having a "tail" 38 that is complementary to nucleic acid strand 34 covalently immobilized at surface 24, is exposed to the surface and dsDNA 36 hybridizes to strand 34 via tail 38.

WO 98/31839 - 22 - PCT/US98/01144

Thus, a single nucleic acid strand is provided that can be covalently linked to surface 24. As used herein, "covarently" is meant to define linkage that is stronger, chemically, than non-covalent linkage such as Van der Waals interactions, ionic interactions, coordinate bonding, and the like. Linkage of species such as X at an appropriate surface, for example thiol linkage to gold, is covalent. The use of component 30 allows nucleic acid strands 34 to be presented at the surface while free of interaction with any neighboring nucleic acid strands. This allows freer access to binding, recognition, and other interaction.

The "nick" 40 in the nucleic acid strand can be mended with DNA ligase enzyme, resulting in immobilized dsDNA species 42 covalently attached to surface 24 via strand 44 extended in the course of the hybridization/ligating step described. When the dsDNA is selected to contain a restriction enzyme site, a restriction enzyme 46 can be used to cut the dsDNA 42. Referring now to Fig. 10, the resulting cut, surface-immobilized dsDNA species 48 can be ligated with any dsDNA that has been cut also with restriction enzyme 46. As illustrated, dsDNA 50 can be hybridized to species 48 and the "nicks" mended with DNA ligase, resulting in surface-immobilized, dsDNA 52 including covalently-immobilized strand 54 and strand 56 which is immobilized via hybridization to strand 54. The "antisense" strand 56 can be dissociated with heat or chemical treatment to expose ssDNA 54 for hybridization studies. Of course, in Fig. 10 strands 54 and 56 include nucleic acids that are not represented throughout the length of each strand.

Thus, the embodiment of the invention represented in Figs. 8-10 includes a single nucleic acid strand immobilized, preferably covalently immobilized, at a surface and not removable from the surface under disassociation conditions. As used herein, the term "disassociation conditions" is meant to define a situation in which, where a single strand 54 is covalently immobilized at the surface and a complementary strand 56 is hybridized to strand 54, strand 56 can be removed. These conditions include hot water, mild chemical treatment, and other techniques available to those of ordinary skill in the art. The invention also includes single strand 54 immobilized to the surface and not removable therefrom under disassociation conditions, and complementary strand 56 hybridized to strand 54 and removable from the surface under disassociation conditions.

Single-stranded or double-stranded nucleic acid can be used to bind, at the surface, biological binding partners that are partners of the immobilized strand or strands, and used in further study. For example, a binding partner of an immobilized strand or strand can be immobilized at the surface, and can serve as a binding partner of yet another biological binding

15

20

partner that then is immobilized, and that species used in studies.

Attachment of a wide variety of nucleic acid strands NA to a moiety R, for example in a way that the strand can biologically bind to its nucleic acid binding partner NAB, can be accomplished with reference to the teaching of examples 10-14, below. It is to be understood that the procedure given in the examples for the preparation of a DNA chip may be applied to the preparation of any nucleic acid chip, such as an RNA chip. Such a chip can be used to detect DNA hybridization (human genome project, diagnostic scanning of DNA for genetic mutants), to present DNA-binding proteins for the study of subsequent protein-protein interactions for which DNA binding is a critical element of the interaction, using instruments such as SPR devices, or to build an easy analysis DNA computer.

The articles of the present invention can be used for a variety of applications, including biosensing applications, test assays, and the like. The term "test assay" generally refers to any procedure in which a member of a biological binding partner pair is to be captured from a medium in which it is dispersed. For example, "test assay" may be used to describe a diagnostic procedure, analytical procedure, microanalytical procedure, forensic analysis, pharmacokinetic study, cell sorting procedure, affinity chromatogram, industrial or laboratory recovery or analysis of one or more species such as toxins, catalysts, or starting materials or products, and the like. a typical test assay is an immunoassay. Biosensing applications include those such as drug screening, environmental monitoring, medical diagnostics, quality control in the pharmaceutical and food industries, and other areas in which it is advantageous to sensitively determine biological binding between partners.

The present invention also provides a method of making an article having a surface for capturing a target molecule. The method involves formulating a solution containing a mixture of at least two self-assembled monolayer-forming species as described herein, and exposing to the surface the solution for a period of time sufficient to form a self-assembled monolayer on the surface. The amount of time required to allow the monolayer to spontaneously chemisorb to the surface will vary depending upon the solvent, the surface, and the monolayer-forming species. Typically, the time required will be on the order of hours, and often a 24-hour exposure is carried out to make certain that maximal coverage is obtained. The degree of formation of a monolayer can be determined by routine spectroscopic methods, as well as physical method in some cases, such as contact angle measurements and the like.

Other methods for forming the monolayer on the surface are included, for example those

20

WO 98/31839 - 24 - PCT/US98/01144

disclosed in copending application serial number 08/131,841, filed October 4, 1994 and entitled, "Formation of Microstamped Patterns on Surfaces and Derivative Articles", incorporated herein by reference.

The embodiment of the invention that involves a SAM-forming species including a nucleic acid strand (or multiple nucleic acid strands) can be used: 1) to detect DNA hybridization (human genome project, diagnostic scanning of DNA for genetic mutants), 2) to present DNAbinding proteins for the study of subsequent protein-protein interactions for which DNA binding is a critical element of the interaction, using instruments such as SPR devices, 3) to build an easy analysis DNA computer. Additionally, both the species X-R-Ch and the species X-R-NA can be used in ultra high through put devices to detect interactions by monitoring the time dependence of electron transfer from bait to prey. The biospecific species self-assemble on a surface such as a metal surface, such as a gold-coated electrode. A peptide or DNA library is generated such that it is also attached to a metal. Specifically, a mixed self-assembled monolayer can be first formed on a gold substrate. The major component is an inert tri-ethylene glycol terminated thiol and the minor component terminates in a photolabile group. Using previously-described masking techniques, a DNA or peptide library is generated such that the spatial address of each species is known. The surfaces are brought into close proximity (atomic force microscopy; AFM technology) so that electrons generating from the gold-tipped electrode travel through a known species immobilized on the electrode via, for example, SAM formation, to a second metal surface, through the surface immobilized library components (peptides or DNA) if and only if there is biological binding between the two. The transfer of electrons from the electrode through individual components of the library can be monitored by a detection array beneath the metal of the library support. Alternatively, a direct interaction can be gleaned from the time dependence of conductance returning to the electrode when an alternating current is applied. The minor component of the self-assembled monolayers can be phenylene-ethynylene thiolates in this embodiment since it has been shown that they have higher conductance than alkanethiolates. However, the two thiols when mixed will form an ordered SAM (See, e.g., Science, "Are Single Molecular Wires Conducting?", Vol. 271, 1996, pgs. 1705-1707).

Referring now to Fig. 11, an electronic biological binding detector system 60 is illustrated schematically, including a first electrode 62 having a surface 64, a second electrode 66 having a surface 68, and an external electrical circuit 70 including a potential source 72 and Ammeter 74 electrically connecting electrodes 62 and 66. Each of electrodes 62 and 66 can be

WO 98/31839 - 25 - PCT/US98/01144

made of a material that also defines surfaces 64 and 68, or can be made of any electrically-conducting material that is coated with a film of material defining surfaces 64 and 68. Where an auxiliary film of material defines surfaces 64 and 68, if the film is thin enough it need not be extremely conductive electrically. In preferred embodiments electrodes 62 and 66 are formed of relatively inexpensive, electrically-conductive material and surfaces 64 and 68 are defined by films of gold. Alternatively, the electrodes can be defined by a supporting substrate of non-conducting material such as glass and coated with a thin film of conductive material such as gold defining surfaces 64 and 68, the films of gold connected to electrical circuit 70.

Each of surfaces 64 and 68 carries a SAM of a major, inert component 30 and a minor, nucleic acid component. Electrode 62 is a probe and carries a SAM including nucleic acid species 70, the sequence of which is known. Surface 68 of electrode 66 carries a SAM including a nucleic acid species 72, a sequence of which is unknown.

When surfaces 64 and 68 of electrodes 62 and 66 are brought into proximity close enough for interaction of nucleic acid strands 70 and 72, for example via AFM, if strand 72 is the complement of strand 70, then hybridization will occur and if the strands are not complementary then hybridization will not occur. Because electron transfer proceeds through hybridized nucleic acid (dsDNA) much faster than through ssDNA, where strands 72 and 70 hybridize, Ammeter 74 will register a distinguishable current flow. This is illustrated in Fig. 12, where strands 70 and 72 are complementary, thus electron transfer can occur to a relatively high degree through the strands and between electrodes 62 and 66. Although electronic circuit 70 is illustrated schematically as a D.C. circuit, an A.C. circuit can be used.

Referring now to Fig. 13, a detection array 80 is illustrated schematically and includes, like system 60, electrodes 62 and 66 having electrode surfaces 64 and 68 upon which SAMs are formed, and external electrical circuit 70. On surface 64 of electrode 62 is formed a SAM 81 of probe ssDNA 82 and inert, major component 30. On surface 68 of electrode 66 is a plurality of regions 84, 86, 88, 100... that together define a matrix of different nucleic acid strands. Each region 84, 86... can be defined by a portion of an overall, continuous SAM across electrode surface 68 (not shown), or can be defined by an isolated SAM island. Each of these arrangements can be constructed in a different manner but may be essentially identical where islands are formed and the intervening regions of the islands filled in with inert species 30. That is, formation of islands and filling in intervening regions with the inert species results in a continuous SAM. In another embodiment, however, individual islands 84, 86... may be defined

by individual regions of patterned gold on the surface 68, the gold regions separated from each other by non-SAM-promoting regions. In this way, discrete islands of SAMs can be formed, separated by areas not carrying any SAM, each SAM being distinct from each other SAM.

In this embodiment, each individual region 84, 86, 88, 100... is addressed by individual electronic circuitry. The illustration is representative in that circuitry addressing region 86, only, is illustrated. When surface 64 is brought into proximity with surface 68, allowing interaction between single nucleic acid strands 82 in SAM 81 of electrode 62 and the SAMs on surface 68 of electrode 66, where the nucleic acid strands hybridize, detectable electron transfer between individual regions of electrode 66 and electrode 62 can be determined. Accordingly, one or more regions 84, 86, 88, 100... can be identified as carrying nucleic acid strands complementary to the probe nucleic acid 82 presented by electrode 62.

The array of different regions 84, 86, 88, 100... of electrode 66 can be fabricated in a variety of ways. According to one technique individual islands of a SAM-promoting material are formed on a non-SAM-promoting surface via photolithography, or other known techniques including etching techniques described in U.S. patent no. 5,512,131, referenced above. In the latter technique a SAM-promoting film, such as a gold film, is deposited on a substrate such as glass, a patterned SAM is formed on the gold surface via "microcontact printing" ("microstamping"), and regions of the gold film not covered by the patterned SAM are etched. Removal of the SAM exposes patterned, isolated gold islands on glass. Alternatively, the microstamped SAM pattern can define regions desirably etched, intervening regions (defining the gold islands desirably preserved) are filled in with a different SAM, and an etchant applied to which the latter-added SAM covering the islands is resistant. Other combinations of techniques for such a process are described in commonly-owned international patent publication no. WO 96/29629, published September 26, 1996, incorporated herein by reference.

In another technique, an array of different SAMs on surface 68 of electrode 66 can be formed by creating a SAM including exposed photolabile groups and, via standard lithographic techniques, creating an array of SAMs as described. In another technique, as described more fully in co-pending, commonly-owned application serial no. 08/616,929, referenced above, an array of SAMs on surface 68 of electrode 66 can be created by positioning a contoured surface of an article against surface 68, the contoured surface creating thereby a series of channels alternating with "blocked" regions on the surface, and filling the channels with a fluid containing a desired SAM-forming species. Manipulation of the article, and use of different articles and

10

20

WO 98/31839 - 27 - PCT/US98/01144

different orientations, as described in the referenced application, can result in creation of the patterned SAM.

The electron transfer detection array is not limited to detection of interaction between nucleic acids, but can be used for any of a wide variety of biological interactions. For example, an array of ssDNA or dsDNA can be created as described, the DNA having a binding region specific for a particular biological molecule such as a protein, and in this manner an array of proteins can be presented on the surface for electron-transfer probing with known species on electrode 62.

Similarly, an array of different species X-R-Ch-M-BP can be formed on surface 68 of electrode 66, a variety of different biological binding partners can be presented at different regions 84, 86, 88, 100..., and region-specific, electron-transfer determination made based upon biological binding between species in the individual, isolated regions of electrode 66 and species presented at surface 64 of the electrode 62.

One particularly suitable application for the species X-R-NA or X-R-NAB, and a chip carrying a SAM of one or more of these, is the study of interacting proteins and protein-DNA complexes that regulate gene transcription. Large soluble yeast PolII holoenzyme/mediator complexes must communicate with some other DNA-bound complex to effect transcription. Precise and accurate determination of interactions of these large complexes with DNA-bound transcription factors would be advantageous, and can be accomplished with the technique of the present invention.

In accordance with an embodiment of the invention in which an article has a surface with a monolayer of X-R-Ch-M adhered thereto, M can serve as a binding partner to capture species that adhere to M. An exemplary list of such species is disclosed in U.S. patent no. 5,250,188, issued October 5, 1993 to Bruening, et al. and incorporated herein by reference.

In all of the embodiments of the invention in which an article is provided, a substrate also may be included in the article. The substrate typically will support a film of material that defines the surface, on a side of the film opposite the side at which a self-assembled monolayer is formed.

The biospecific SAMs described herein afford several advantages that make the technique of this example sensitive and practical: 1) they self-assemble on metal - one of the factors necessary for electron conduction; 2) because they are mixed monolayers with the biospecific thiol being the minor component, the ligands of interest can be immobilized far enough from

20

each other that electron transfer will not occur radially in the plane of the electrode surface which would compromise and complicate results; 3) the rigid structure of a SAM ensures that the immobilized ligands are held a fixed distance from the electrode which is important because measurements of the electron transfer rates depend on the distance the electrons travel; 4) the biospecific SAMs control the orientation of the immobilized molecule (active site or DNA orientation), clear advantage over random coupling procedures that would put the site of interaction at varying distances from the electrode complicating and compromising results; 5) biospecific SAMs provide a universal acceptor surface to which any unmodified oligo or histidine-tagged protein can be attached. 6) the aliphatic chain of the biospecific thiol can be modified (Bumm, L.A., Arnold, J.J., Cygan, M.T., Dunbar, T.D., Burgin, T.P., Jones, L.II, Allara, D.L., Tour, J.M., Weiss, P.S., Are single molecular wires conducting?, Science 271 (1996) pp. 705-707) so that it is much more conducting than the inert major component thiols thus giving a greater signal:noise ratio. Phenylene-cthynylene benzothiolates have been shown by Bumm, et al. to be much more conductive than alkanethiolates. Mixed SAMs of the two species could be formed. The described array-array interface system can easily be scaled up giving ultra high through-put detection.

In the embodiments discussed and illustrated above, in particular in connection with Figs. 8-13, electron transfer is used as a tool to determine biological binding. In another set of embodiments applicable to the systems illustrated in Figs. 8-13, an electronic property not necessarily defined by electron transfer can be used to determine biological binding and other chemical interaction. For example, the systems illustrated in Figs. 8-13 can involve, rather than direct current probing across biological molecules, alternating current probing of the molecules for determination of a shift in phase of alternating current applied to the system. In the embodiments illustrated, power source 72 would be replaced by a source of alternating current, and ammeter 74 would be replaced by a phase detector.

One system 90 for electronic-property probing of molecules is illustrated schematically in Fig. 14. System 90 is a one-electrode system including an electrode 92 and a container 94 defining an enclosure, one surface of which is defined by a surface of electrode 92. Container 94 includes, as illustrated, a fluid inlet 93 and a fluid outlet 95. Container 94 need not be a fluid enclosure, as illustrated, but need only be capable of positioning a medium containing a target species 98 in proximity of electrode 92. Electrode 92 includes a surface 100 upon which is a SAM 102 including NSB-minimizing species 30 and species 104 defined by a species X-R-Ch,

20

X-R-Ch-M-BP, X-R-NA, all as described above, or any other SAM-forming species terminating in a chemically or biologically-active species. "Chemically or biologically active species", as used herein, is meant to include proteins, enzymes, nucleic acids, species that engage in phosphorylation or hydrolysis, species that undergo conformational changes, photochemically-active species, electrochemically-active species, and the like. The ratio of species 104 to 30 can be as described above. Species 104 terminates in the chemically or biochemically active species, that is, the end of species 104 facing away from the surface 101 of electrode 92 terminates in the actives species.

Electrode 92 can be a STM tip, or the like. The electrode can be gold-coated with a thiolend group containing SAM-forming species defining SAM 102.

Target species 98 introduced into container 94 can be any analyte of interest, for example a protein, DNA, RNA, other nucleic acid, small molecule, catalyst such as enzymes, affector molecule, and the like. The medium within which target species 98 is carried can be a fluid selected solely for the purpose of delivering the target molecule to the electrode. It me medium can have electrical conductivity or resistivity if desired. It can be useful for the fluid medium to be conductive in the case of all DNA hybridization. Electrically-nonconductive fluid media would be useful in a phase-shift detection system where the particular degree of conductivity can be selected for a particular application.

Species 104 and species 98 are chemically or biochemically active with respect to each other. Thus, when a reaction is allowed to take place involving species 104 (for example biological binding between target species 98 and species 104), a change of an electromagnetic property of the system results. For example, if species 104 and 98 are protein/protein, protein/DNA, or DNA/DNA (including other nucleic acid binding partners), and if binding occurs, or where species 98 is a species that causes a structural or chemical modification of immobilized species 104, then detection can be made by applying an alternating current to the electrode and monitoring changes in electromagnetic properties of the system such as changes in voltage, current or impedance as a function of frequency and/or temperature. Binding of species 98 to species 104, or a chemical or structural change of species 104 caused by species 98 will cause a characteristic detectable electrical property signal in the system such as a change in impedance or dielectric value.

As illustrated, the system includes an external electrical circuit 91 including a source 97 of alternating current, and an impedance sensor 99. The system is grounded. The impedance

RECTIFIED SHEET (RULE 91) ISA/EP

ŧ

10

20

25

sensor can be a current sensor in parallel with circuit 91, or another arrangement, such arrangements being within the level of ordinary skill in the art.

Electronic properties that can be sensed in the systems of the invention to determine biological or chemical interaction include impedance (a combination of capacitance, inductance, and/or conductance) dielectric properties of the system (which can be derived from others of these properties listed) and the like. It is necessary only that a detectable change in electronic configuration occur. "Electronic configuration" in this context means electron transfer or other shift in electrons within a species that produces a detectable change in electronic property (such as impedance, above, creation of dipoles, and the like).

The system of Fig. 14 can operate as follows. A fluid is introduced into inlet 93 and fills container 94, exiting at outlet 95. The impedance of the system is measured, and if biological interaction between species 98 and species 104 takes place, then changes in the impedance of the system will be determined indicative of interaction of species 98 and species 104.

Determination of electronic properties of molecules can be carried out using a twoelectrode system as well, and such a system 110 is illustrated in Fig. 15. System 110 includes, in
addition to electrode 92 and SAM 102, a second electrode 112 spaced from electrode 92.
Electrode 12 can be the same as or different from electrode 92. Both electrodes can be
conductive or semiconductive. The system includes a medium 114 between electrodes 92 and
112, which can be introduced into container 94 through inlet 93 and removed through outlet 95.
The medium can be conductive, non-conductive, or semiconductive electrically. When medium
114 is non-conductive, the system can define a capacitor. Electrode 112 does not carry a SAM,
or carries a SAM (not illustrated) made up of nonactive species, that is, species that are not
chemically or biochemically active with respect to species 98. For example, a SAM of NSBinhibiting species 30 can be provided on electrode 112 if desired.

Electrical circuit 116 is provided to electrically connect electrodes 92 and 112, and includes a source 97 of alternating current and an impedance sensor 99. In another arrangement (not illustrated) impedance is sensed by providing a source 97 or alternating current that addresses electrodes 92 and 112, a current meter in parallel with that circuit, and a voltage meter arranged, separately, across electrodes 92 and 112. This arrangement could be used also in connection with Fig. 16, described below.

The system 110 of Fig. 15 operates as follows. Species 98 is introduced into the system. If binding to species 104, or chemical or biochemical modification of species 104 occurs, then

10

20

WO 98/31839 - 31 - PCT/US98/01144

this binding is sensed by monitoring electronic changes in the real or imaging parts of impedance (including wave properties and wave echos) of the system as a function of frequency, time, and/or temperature. Electronic sensing of molecular processes also can be carried out in a two-electrode system where both electrodes present functionalized SAMs, that is, where both electrodes provide a SAM including a species that can biologically or chemically interact with a species in solution or with species of the other electrodes. This can be visualized with respect to Fig. 12, above. When the two electrodes are mechanically brought into close proximity so that interaction of the separately immobilized species occurs, the impedance of the system is changed. These changes in impedance can be detected by comparison of high frequency response profiles where changes in impedance, dielectric, or conductance as a function of frequency, time, and/or temperature are monitored. Reference can be made to Heller, "Electrical wiring of redox enzymes", *ACC. Chem. Res.* 23, 128-134 (1990) and Bumm, et al., "Are single molecular wires conducting?", *Science*, 271 (1996), referenced above.

The frequency range for detection associated with the system of Fig. 15 should be in the Khz to gHz frequency range. At high end frequencies impedance can be dominated by water (if present). At the low end of these frequencies, conductivity across the medium, or capacitance of the electrode itself, can dominate, preventing determination of a change in electromagnetic properties due to chemical or biochemical activity of species 98 with respect to species 104.

to system 110 with the exception that a first electrode 122 is provided including a SAM 124 defined by species 30 and, admixed with species 30, SAM-forming species 126 defining double-stranded DNA exposed at the surface. Species 128 is exposed to SAM 124 (by, for example, introduction in a fluid medium into inlet 93), and a change in chemical, biochemical, or structural characteristic of species 126 is determined. In the embodiment illustrated, species 128 (the target species) is a drug that functions in its ability to intercalate into duplex DNA (Blasko et at., "Microgonotropens and their interactions with DNA: structural characterization of the 1:1 complex of D(CGCAAATTTGCG)₂ and Tren-microgonotropin-b by IID-NMR Spectroscopy and Restrained Molecular Modeling", *J. Am. Chem. Soc.*, 116, 3726 (1994)). Species 128, which is a small molecular drug candidate, is injected into the system and changes in the structure of the system (i.e. the interruption of even one base pairing of DNA 126) cause a change in the electromagnetic properties at the surface. As the small molecular species (drug 128) intercalates into duplex DNA, the dielectric of the system is altered due to structural changes even though the

WO 98/31839 - 32 - PCT/US98/01144

molecular content of the system has not changed (Sridhar, et al., "High-frequency structural relaxation in supercooled liquids", *J. Chem. Phys.* 88, 1170 (1988)). The structural alterations (and, by implication, the action of the drug candidates) can be detected by monitoring changes in the dielectric (impedance) of the system as a function of frequency and/or time.

In another arrangement (not illustrated) a SAM on a surface of an electrode is provided. The SAM is made up of an active species, optionally (and preferably) in combination with species 30. A species is introduced, and immobilized to the active species of SAM. A second species is introduced and determination is made as to whether the second species displaces the first species coupled to the SAM-forming species. Detection can be carried out as described above involving impedance.

In another procedure, DNA hybridization and analysis of base pair mismatch (for screening genetic mutations) can be carried out. A DNA SAM is provided on an electrode, a complementary strand is exposed to immobilized DNA, and where mismatch occurs impedance or conductance is changed detectably. Determination can be made as to where in the sequence the mismatch occurs, and the identity of the mismatch, by measuring the impedance of the system as a function of frequency.

In another example, a coaxial cable defines an electrode used in a determination. The use of the known property of and modification of a coaxial cable is used. The invention, according to this embodiment, involves modification of the end of the cable with a SAM, as described above. The cable, once the end is modified, can be coupled to a network analyzer used to scan frequencies to eliminate wave reflections. Hookup of the analyzer, and comparison of the analyzer to "before" and "after" measurements (before and after exposure of the end of the cable to a medium containing a species that can chemically, biochemically, or structurally alter a species of the SAM) results in determination of the interaction. That is, wave reflection values can be compared between "before" and "after" characteristics.

As discussed, electronic sensing strategies include determination of and the modification of an immobilized species. Modification can be caused by a second species, by light, by a magnetic field, or other phenomena. One example of a chemical change is immobilization of ATP on a SAM-derivatized electrode. A catalytic substance is introduced into a conducting medium between two electrodes (for example, the arrangement as illustrated in Fig. 16). The eatalyst induces change of ATP to ADP as an indicator of a biological function. This is detected by monitoring the change in impedance of the system.

30

5

7()

Another aspect of the invention involves determination of a mis-folded form of a normal protein or any induced conformational change of a normal biological molecule. Determination can be made via systems such as those illustrated in Figs. 8-16, or in connectiong with embodiments illustrated in Figs. 17 and 18, discussed below.. This embodiment can be useful in detection of changes in state of oligomerization of a biological molecule. Many disease states (including a form of breast cancer, for example) involve a receptor that is too prone to dimerization, either by overproduction of a ligand that promotes receptor dimerization or a mutation in the receptor itself. A disease state results when the dimerization state sends a constituitively "active" signal. Drugs capable of disrupting this dimerization (or, inconnection with some diseases, promoting it), could be screened in this embodiment of the invention. Referring to Fig. 17, this can be screened in device 300 including a first electrode 302, a second electrode 304, and an electrical circuit 306 connecting electrodes 302 and 304 and including a source 308 of alternating current and an impedance sensor 310. A SAM 312 includes NSBinhibiting species 30 and an immobilized (preferably covalently via attachment to a SAM forming species) ligand 314. A receptor as a multimer 316 is immobilized at ligand 314 and a drug candidate 318 is introduced into a compartment 320 through an inlet 322. The drug candidate 318 is capable of disrupting the multimer 316. Receptors, free in solution, can be subjected to drug candidates that might promote dimerization which can result in an altered electronic characteristic (impedance) state of the system.

This invention can be used, in particular, in connection with detection of disease states involving mis-folded forms of normal proteins, e.g. transmissible prion diseases (TSE's). This includes disease states such as transmissible spongiform and encephalopathies (TSEs) such as bovine spongiform encephalopathy (mad cow disease), scrapie in sheep or in humans, Creutzefeldt-Jacob disease, fatal familial insomnia, Gerstmann-Straussler-Sheinker disease, kuru, and the like. Those of ordinary skill in the art are aware of this type of disease and what is embraced by the designation "disease states involving a mis-folded form of a normal protein". These disease states are believed to occur when a mis-folded form of a normal protein (prion) enters a mammal (see "Special News Reports Putting Prions to the Test" *Science*, 273:184-189, 1996). In a host animal, the prion is proposed to propagate a disease state by recruiting normal proteins and catalyzing the mis-folding of the normal protein so that the normal protein, in turn, goes on to infect other normal cells and tissues of the host mammal. A long-standing problem in the art is the inability to effectively detect disease states involving mis-folding of normal

15

proteins. Typically, in the prior art, disease states of these types can be detected only in a late stage of the disease. The disease typically is first detected clinically by behavioral changes in a mammal, and is verified after death of the mammal by visual inspection of defects in the brain (sponge-like holes). However, carlier detection of this type of disease state would be extremely beneficial since the prion is very resistant to protease and heat treatment, and remains infectious unless treated with harsh organic solvents. Because of the great risk of transmission to humans or other mammals from tainted meat or feed (even if cooked) pre-clinical diagnosis has become a significant area of research, although prior to the present invention, simple and reliable preclinical diagnosis has not resulted. The present invention provides simple, pre-clinical diagnosis involving obtaining a fluid sample of a mammal e.g., blood, tears, a non-invasive tissue sample such as a needle biopsy of a tonsil, tissue homogenates, and the like, and subjecting the sample to a test to determine the presence of prion.

Referring to Fig. 16, this can be envisioned by use of system 120 where SAM 124 includes NSB-resistant component 30 in combination with a SAM-forming species analogous to species 126 but to which normal protein is immobilized covalently, or via the polyamino acid tag/chelate arrangement described above, or the like. Alternatively, the entire SAM can be made up of immobilized protein, but it is preferred that the SAM included NSB-resistant species 30 be present according to proportions described above. Exposure of this system (through inlet 93 to a fluid sample containing a fluid or tissue sample of a mammal suspected of containing a TSE disease state, if the disease state is present, it will result in mis-folding of the normal protein immobilized at the surface via the SAM, resulting in a change in the electronic configuration of the immobilized protein indicated by a detectible change in electrical properties of the system. such as a change in impedance of the system (capacitance). The change in conformation of the immobilized protein induced by the prion or associated cofactor (which is indicative of the disease state) is detectible. Prior studies have been conducted involving determining a change in material conformation of a molecular species via a detectible change in capacitance between a capacitor. Specifically, glass in a liquid state and in a solid state, in a capacitor, has been studied and the change between liquid and solid has been detected via a change in capacitance. This change in electrical property is due to a change in material confirmation alone. The Applicant is not aware of prior studies that have taught or suggested application of this technique to biological systems.

This aspect of the invention is particularly useful since it makes otherwise very difficult

RECTIFIED SHEET (RULE 91) ISA/EP

WO 98/31839 - 35 - PCT/US98/01144

detection of a change in conformation of a biological molecule possible. Prion detection has been difficult in the past because the amino acid sequence of the normal and the mis-folded proteins are identical. The normal protein has been proposed to be predominantly an α -helical while the prion form is a β -sheet. Early detection is very important in live stock since, for example, scrapie has an incubation period of about two years before presentation of clinical systems and, in the interim, great expense to raise the animal is wasted.

Changes in capacitance due to liquid/solid change in glass is described by Sridhar et al., "High Frequency Structural Relaxation in Supercooled Liquids" *J. Chem. Phys.* 88, 1170-1176 (1988).

In another aspect the invention involves a spacially-addressable surface array of biological molecules, i.e., a "combinatorial" array in which each individual region of the array is individually addressable electronically, and each can serve to detect, via determination of a change in electronic property of a surface-immobilized species, a chemical or biochemical interaction at that surface. This can be envisioned with reference to Fig. 13 in combination with Fig. 16. An array as illustrated in Fig. 13, created via micro contact printing, via formation of a thin film of metal such as gold on a surface followed by scoring of the surface to electronically isolate individual regions of the film, or the like can be provided. Each individual, isolated metal region serves as an electrode and is individually addressable, for example through the substrate. In another embodiment, photo lithographic techniques or micro contact printing in combination with plating or etching can result in an array of individually-addressable electrodes each addressed by a lead on the top surface 68 of electrode 66 of Fig. 13. Electrode 66 of Fig. 13, in combination with an electrode such as electrode 124 of Fig. 16 (that can be free of any SAM) can be provided in combination, or the electrode carrying the combinatorial array can be provided alone as in Fig. 14. The use of combinatorial arrays is known (see "Special Report: Combinatorial Chemistry" Chemical & Engineering News, February 12, 1996, page 28+). In the invention, individual chemical or biochemical species are provided at individual, isolated electrode regions of the array, the array is exposed to a medium suspected of containing a species that interacts with at least one immobilized chemical or biochemical species, and a change in electronic property of a surface-immobilized species indicates interaction at the relevant electrode of the array, identifying the surface-immobilized species involved in the reaction, thus identifying the species to which the surface was exposed. Essentially any chemical or biochemical interaction can be determined in this manner, for example mass screening of drugs,

10

15

and the like.

One advantage of this system is that the determination is not only qualitative, but quantitative. That is, not only can interaction of a species immobilized at the surface with a species to which the surface is exposed be detected, but the degree of interaction scales with the degree of change of electronic property at the surface, thus the degree of interaction can be determined, not just the existence of the interaction.

This aspect of the invention is a significant improvement over the state of the art as known to the applicant in which random sequences of peptides are synthesized on beads, on a spacially addressable surface (such as combinatorial array) or displayed by phage. Target proteins are tagged with a visual marker (usually a fluorescent tag) and the beads or surfaces are incubated with the target molecule to allow for binding. After several washing steps, beads that fluoresce are isolated or the positions on the surface that fluoresce are identified. The sequence of a peptide in a spacially addressable array that interacts with the target molecule is determined, in the prior art, by a photo-ma king scheme. The sequence of peptides on beads that interact is identified by micro-sequencing techniques. One drawback of the prior art techniques is that, in affinity studies of small peptides, degradation in the body, diversity, and representation are problematic. If a peptide in a study is provided as an 8-mer (eight peptide units) the peptide is not large enough to provide the structure necessary for accurate binding determination. On the other hand, if a larger peptide (e.g., a 20-mer) is used, better structural integrity exists and a better chance of binding exists. However, this option involves under-representation since one would need approximately 1025 different peptides for all possibilities to be screened. It is not feasible to make and screen random 20-mers and barely feasible for 10-mers. The usual length of a randomly synthesized peptide is an 8-mer which, in many cases, is unacceptable conformationally. Additionally, a peptide that is small enough to be readily synthesized and screened typically is degraded by the mammalian body too quickly to compete with native 25 proteins.

The present invention solves this problem by using cyclic compounds, currently studied as denovo structural elements, but not, according to the applicant's knowledge, for drug discovery, in an inter-connected linkage defining an unnatural polymer. The technique involves, by a photo-synthetic and photo-masking technique, synthesizing a spacially addressable surface array (Fig. 13) of a SAM including, at each individual array location, an immobilized putative binding partner 200, as illustrated in Fig. 18. Species 200 includes amino acids 202, 204, 206

RECTIFIED SHEET (RULE 91) ISA/EP

WO 98/31839 - 37 - PCT/US98/01144

and 208, linked to cyclic structures. Cyclic structures of this general type have been described by Gellman and Kiessling, albeit without any teaching or suggestion of attaching functionalities to them. Each individual molecule 200 is different, and is provided at a location of the combinatorial array. The surface is incubated with a putative binding partner, then washed. The added mass of molecules that interact with the surface cause changes in the electronic properties of the system. The spacial location of the surface-immobilized species that interact with the target molecule can be identified electronically since the sequence of the immobilized species is pre-determined by a masking scheme; its physical location in the array identifies the sequence.

Species 200 (Fig. 18) also can be an oligonucleotide such as DNA including unnatural bases derivatized with functionalities for the acceptance of modifying molecules (bases available from Glenn Research). The amino acids can be attached to these bases. In this case the oligonucleotide provides the structure, and the amino acids provide specificity.

This system also can be applied to beads or other supports and incubation can take place with a fluorescently-tagged target protein. Binding candidates can be visualized and then isolated.

The immobilized species to be synthesized can be a simple peptide or nucleic acid. Alternatively, it can be a polymer of ring compounds connected by amide bonds, as illustrated in Fig. 18, or pseudo peptide bonds such as a vinylogous backbone (Gellman, *Nature*, August 1996). Gellman describes ring compounds connected by amide bonds that are protease resistant and also form repetitive tertiary structures. The cyclic polymers can be synthesized in much the same way as a peptide. The rings can easily be modified with a functionality to accommodate the attachment of an amino acid. The resultant pseudo peptide gains rigidity from the ringed structures and specificity from the amino acid. A rigid scaffold for the presentation of amino acids simplifies the diversity and representation problem. If the rigidity from three amino acids can be replaced by one ring compound then the synthesis problem is simplified by a factor of 8,000. In this case, a 20-mer can be represented by four ring compounds plus eight amino acids. Five amino acids that contribute to structure can be eliminated.

DNA-containing SAMs can be used for the identification of randomly-sequences peptides (natural or unnatural) or small molecule polymers. Random synthesis of peptides or small molecule polymers can be carried out on beads or on other supports and each synthetic step tagged by a four-digit DNA code. Drug candidate beads can be incubated with a tagged target protein. The sequence of an interacting species can be identified by passing the bead over a

30

DNA-SAM array. The code and thus the sequence of the polymer is identified by visualization or electronic detection of hybridization to the complementary DNA presented by the SAM.

The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples below. The following examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope of the invention. References cited in the following examples are incorporated herein by reference for all purposes.

Example 1: Preparation of Nitrilotriacetic Acid Chelate Linked Via Spacer Moiety To Thiol

Undec-1-en-11-yl tri(ethyleneglycol) (1) was synthesized according to a procedure reported by Pale-Grosdemange, C.; Simon, E.S.; Primc, K.L; Whitesides, G.M. <u>Journal of the American Chemical Society</u>, 113, 12, (1991).

(1) $H_2C=CH_2(CH_2)_9-(OCH_2CH_2)_3-OH$

N-(5-amino-1-carboxypentyl)iminodiacetic acid (2) was synthesized according to a procedure reported by Hochuli, E.; Döbeli, H.; Schacher, a. <u>Journal of Chromatography</u>, 411, 177 (1987).

(2) $H_2N-(CH_2)_4-CH(CO_2H)(N(CH_2CO_2H)_2)$

Carbonyldiimidizole (2.3 g, 2 eq. was added to a stirring solution of 2.2 g of alcohol (1) dissolved in 25 ml methylene chloride. After stirring for 2 hours, the solution was applied to a 300 g column of silica equilibrated with ethyl acetate and the imidazole carbamate cluted with 1 liter of ethyl acetate. Evaporation of the solvent under reduced pressure gave 2.7 g (95%) of the imidazole carbamate as an oil.

Amine (2) (5.0g) was dissolved in 100 ml of water. The pH was titrated to 10.2 with 12 N NaOH, then 130 ml of dimethylformamide was added. The imidazole carbamate (2.5 g in 10 ml dimethylformamide) then was added dropwise to aqueous solution of (2) while stirring. After 12 hours, the solution was added to 500 ml water and washed three times with ethyl acetate by gently stirring to avoid the formation of an emulsion. The aqueous phase then was acidified with 6 N HCl to pH 1.5 and extracted into ethyl acetate (4 x 250 ml). The combined extracts were

10

15

20

washed with saturated NaCl, dried over MgSO₄, and the solvent removed under reduced pressure to give 1.8 g (50%) of olefin (3) as a hydroscopic white solid.

 $(3) \ \ H_2C = CH_2(CH_2)_{9} - (OCH_2CH_2)_{3} - O(CO)NH_2(CH_2)_{4} CH(CO_2H)(N(CH_2CO_2H)_2)$

To olefin (3) (1.7 g) dissolved in 15 ml distilled tetrahydrofuran was added 0.5 ml thiolacetic acid and 100 mg 2,2'-azobis(2-methylpropionitrile) (AIBN). The solution was irradiated for four hours under a 450 W medium pressure mercury lamp (Ace Glass). The solvent was removed under reduced pressure and the crude product triturated with hexane. Recrystalization from ethyl acetate/hexane gave 1.8 g (94%) of thioacetate as a hydroscopic tan solid (4).

$(4) \ \ H_3C(CO)S-(CH_2)_{11}-(OCH_2CH_2)_3-O(CO)NH-(CH_2)_4CH(CO_2H)(N(CH_2CO_2H)_2)$

To thioacetate (4) (0.67 g) in 20 ml dimethoxyethane was added 17 ml water, then 20 mg I_2 . After the addition of 3 ml of 2 N NaOH, the solution was stirred for four hours while bubbling with O_2 . Addition of 100 ml of water and acidification to pH 1.5 with 6N IICl lead to precipitation of the product as the disulfide. The disulfide was filtered, washed with water and dried under vacuum to give 0.55 g (89%) of a white powder.

The disulfide was reduced to the thiol (6) with triethylphosphine. To the disulfide (0.42 g) in 18 ml methanol plus 2 ml water under an atmosphere of nitrogen, was added 0.3 g of triethylphosphine. The solution was stirred for five hours then concentrated to an oil under reduced pressure. The residue was dissolved in 30 ml degassed water and acidified to pH 1.5 with 6N HCl. The product was extracted three times with 20 ml ethyl acetate. The combined organic phases were washed with saturated NaCl, dried over MgSO₄, and concentrated under reduced pressure to thiol-linked nitrilotriacetic acid chelate (5) as a tan hydroscopic solid (0.24 g, 57%).

 $(5) \ \ HS-(CH_2)_{11}-(OCH_2CH_2)_3-O(CO)NH-(CH_2)_1-CH(CO_2H)(N(CH_2CO_2H)_2)$

Example 2: Preparation of SPR Chip Derivatized With Self-Assembled Mixed Monolayer of Chelate And Nonspecific Binding Inhibitor

11-mercaptoundec-1yl oligo(ethyleneglycol) (6) was synthesized according to a

30

5

15

procedure reported by Pale-Grosdemange, et al., <u>JACS</u> (1991; above).

(6) $HS-(CH_2)_{H}-(OCH_2CH_2)_n-OH$

5

15

20

25

The gold surface of an SPR chip from Pharmacia Biosensor, Piscataway, NJ was cleaned using 70:30 H₂SO₄/H₂O₂ to remove all species on the gold. 11-mercaptoundec-lyl oligo(ethyleneglycol) (6) and the thiol-linked nitrilotriacetic acid chelate (5) described in example 1 were dissolved in ethanol in a 95:5 molar ratio, at a total concentration of 1 mM. The chip surface was exposed to the 1mM solution of the thiols in ethanol for 24 hours, then washed with 95% ethanol and allowed to dry. The derivatized surface was characterized by X-ray photoelectron spectroscopy, which revealed that a mixed monolayer had adhered to the surface, the mixed monolayer including a ratio of (6) and (5) approximately equal to the concentration of those species in the solution from which the surface was derivatized (95:5 molar ratio of (6) to (5)).

Example 3: Incorporation of Metal Cation Into Chelate of SelfAssembled Monolayer on SPR Chip

The surface derivatized as described in example 2 to have adhered a self-assembled mixed monolayer formed of a 95:5 molar ratio of species (6) and (5) was further modified to allow the chelate to coordinate nickel dication (Ni²⁺). The mixed monolayer was washed with 1 mM aqueous NaOH followed by 1% aqueous Ni(SO₄).6H₂O. resulting in species (5) coordinating Ni²⁺ to define species (7).

 $(7) \ \ HS-(CH_2)_{11}-(OCH_2CH_2)_3-O(CO)NH-(CH_2)_4-CH(CO_2H)(N(CH_2CO_2H)_2).Ni^{2+}$

Example 4: Coordination of Biological Binding Partner To Metal Cation

Gal 11 has been identified as a protein required for efficient galactose utilization in yeast. Nogi, Y.; Fukasawa, T. <u>Curr. Genet.</u> 2, 115-120 (1980). Gal 11p (potentiator) is a 44 kD mutant of Gal 11, differing from Gal 11 at a single amino acid only <u>Id.</u> Gal 11 is a suspected biological binding partner of Gal 4 (see discussion in example 5). a "mini" form of Gal 11p was expressed and purified (residues 261-351 + 799-1081) with a polyamino acid tag at a location remote from the recognition region of the protein

fragment. Specifically, a run of six histidines is expressed at the amino terminus of the protein fragment, and the histidine-tagged Gal 11p was isolated out of cell extracts by absorption on a column containing nickel dication coordinated to a nitrilotriacetic acid derivative. Hochuli, et al. <u>J. Chromatog.</u> 411, 177 (1987), Gentz, R.; Chen, C.; Rosen, C. a.; Proc. Natl. Acad. Sci. 86, 821 (1989).

The polyamino acid-tagged binding partner was attached selectively to the derivatized surface described in example 3 by coordination to the metal cation via the histidine tag. specifically, a 0.25 mg/ml aqueous solution of Gal 11p was injected into a delivery channel of a BIAcoreTM SPR instrument, Pharmacia Biosensor. The result was a surface carrying a plurality of binding partners (Gal 11p) oriented such that a substantial portion of the recognition regions for Gal 4 face away from the chip surface and are readily available for biological binding with the biological molecule Gal 4.

Example 5: SPR Determination of Gal 4 Using Chip Derivatized With Self-Assembled Monolayer of Alkyl Thiol Terminating In Metal Chelate Coordinated By Polyamino Acid-Tagged Gal 11p

This example examines the sensitivity of the SPR technique for the analysis of the interaction between biological binding partners, with use of an SPR chip prepared in accordance with the present invention. Specifically, the interaction between the transcription promoter Gal 4 and Gal 11p (which contains increased area of hydrophobicity relative to Gal 11), was studied, using a BIAcoreTM SPR instrument available from Pharmacia Biosensor, Piscataway, NJ.

It has been reported that in cells deleted for Gal 11, activation by Gal 4 is reduced some 5-20 fold, an effect that can not be accounted for by decreased levels of the activator. Id, Himmelfarb, H. J.; Pearlberg, J.; Last, D. H.; Ptashne, M. Cell 63, 1299-1309 (1990), Suzuki, Y.; Nogi, Y.; Abe, a.; Fukasawa, T. Molecular and Cell Biology 8, 4991-4999 (1988), Long, R. M.; Mylin, L. M.; Hopper, J. E. Molecular and Cell Biology 11, 2311-2314 (1991). It is suspected that proteins containing the Gal 4 region that recognizes Gal 11p in vivo interact in vitro with Gal 11p but not with Gal 11 (wild type).

It is also suspected that Gal 4 promotes transcription by facilitating the necessary proximity of a holoenzyme containing Gal 11 with the TATA box on DNA. It has been reported that Gal 4, truncated to completely abolish its activating domain, is

30

transcriptionally active in the presence of the holoenzyme containing Gal 11p (but not in the presence of the holoenzyme containing Gal 11). Accordingly, it has been postulated that Gal 11p interacts hydrophobically with truncated Gal 4 to facilitate transcription.

SPR was used to study the interaction of Gal 4 with the immobilized his-tagged Gal 11p. It was observed that Gal 11p binds Gal 4, but does not bind a number of other transcription promoters. Histidine-tagged wild-type Gal 11, immobilized on the SPR chip in the same manner, did not bind Gal 4. None of the transcription promoters demonstrate NSB on the mixed monolayer. That is, in the absence of Gal 11p immobilized on the surface, no transcription promoters bound to the surface, and in the presence of immobilized Gal 11p, only Gal 4 was bound.

The interaction between Gal 11p and Gal 4 was not observed by co-immunoprecipitation or on an affinity column, demonstrating the importance of the orientation in the presentation of the protein to the analyte solution, achievable in accordance with the present invention.

15

20

25

10

Example 6: Comparative Attempted SPR Determination of SRB2 Using Standard Dextran Chip Carrying Gal 11

This example examines the sensitivity of the current state-of-the-art SPR chip, which comprises a layer of dextran on the gold SPR chip surface.

In this example, an attempt was made to study the interaction of Gal 11 (wild-type) and the RNA polymerase B suppressor SRB2, a 28 kD member of the holoenzyme described in example 5 (the multi protein complex which assembles on the DNA at the TATA box and with which a DNA-bound activator must interact in order to activate transcription). Koleske, a. J.; Young, R.A. Nature, 368, 466-469 (1994). As discussed in example 5, Gal 11p interacts with Gal 4, thus it is important to find a target of Gal 11 on the holoenzyme. Detection of this type of interaction is difficult because transcription factors generally exhibit low affinity for their targets, such that positive control of transcription of achieved only through the correct assembly of several factors. The 30 kD protein SRB5 was used as a negative control in this example. SRB5 is shown by genetic experiments not to interact with Gal 11 or Gal 11p Id.

An SPR chip having a layer of dextran on gold (#BR1000-14) was purchased from Pharmacia Biosensor., and the SPR instrument described above was employed.

Fig. 1 illustrates a sensorgram plotting resonance units (RU) as a function of time associated with this example. The flow rate of sample across the chip surface was 5 ul/min. The sensorgram of Fig. 1 is labeled with reference numerals that correspond to the steps of the experimental protocol below.

5

10

20

- 1. (t=240 sec) description of chip type; standard dextran
- 2. (t=731 sec) end of injection of EDC/NHS which activates the carboxylates on the dextran surface.
- 3. (t=13.26 sec) end of injection of 35 ul wild type Gal 11 at 0.025 mg/ml, in NaOAc buffer at pH 4.5.
 - 4. (t= 1440 sec) preinjection baseline
- 5. (t=1928 sec) end of injection of ethanolamine, which blocks the activated carboxylates that did not covalently link to a protein.
 - 6. (t=2179 sec) preinjection baseline
- 7. (t=2668 see) end of injection of SRB2 at 0.25 mg/ml diluted with the running buffer PBS.
 - 8. (t=2991 sec) preinjection baseline
 - 9. (t=3472 sec) end of injection of SRB2 at 0.5 mg/ml
 - 10. (t=3639 sec) preinjection baseline
 - 11. (t=4122 sec) end of injection of SRB5 at 0.25 mg/ml
 - 12. (t=4218 sec) preinjection baseline
 - 13. (t=4814 sec) end of injection of SRB5 at 0.5 mg/ml
 - 14. (t=5610 sec) preinjection baseline
- 15. (t=6128 sec) end of injection of myc-a, a monoclonal antibody which should bind to Gal 11, at 0.25 mg/ml.

Tabulated below are protein baserption response values (AbsResp; RU units) and response values relative to preceding baseline (RelResp; RU units) corresponding to the protocol steps above.

	WO 98/31839		- 44 -	PCT/US98/01144
		<u>Time</u>	<u>AbsResp</u>	RelResp
	1.	240.00	9150	
	2.	731.00	9313	
	3.	1326.50	13444	4131
5	4.	1440.00	13291	3978
	5.	1928.00	12950	3637
	6.	2179.00	12872	3559
	7.	2668.00	12923	51
	8.	2991.00	12880	8
10	9.	3472.00	12976	96
	10.	3639.00	12895	15
	11.	4122.00	12911	17
	12.	4218.00	12887	-8
	13.	4814.00	12941	54
15	14.	5610.00	12852	-35
	15.	6128.00	12940	87

4131 RUs of Gal 11 bound to the dextran. The interaction of Gal 11 with SRB2 yielded an absorption of 51 RU's at [SRB2] = 0.25 mg/ml; at [SRB2] = 0.50 mg/ml, the absorption was 96 RUs. By contrast, at [SRB5] = 0.25 mg/ml the absorption was 17 RU's. while at [SRB5] = 0.5 mg/ml, the absorption was 54 RUs. Lastly, at [myc-a] = 0.25mg/ml, the absorption was 87 RU's.

Since the negative and positive controls (SRB5 and myc-a, respectfully) gave essentially the same results, no conclusions regarding binding can be reached. This example demonstrates that the current state-of-the-art dextran chip does not provide the sensitivity necessary for determination of biological binding between Gal 11 and SRB2.

Example 7: SPR Determination of SRB2 Using Chip Derivatized With Self-Assembled Monolayer of Alkyl Thiol Terminating In Metal Chelate Coordinated By Polyamino Acid-Tagged Gal 11

As in example 5, this example demonstrates the sensitivity of the SPR technique using a chip derivatized in accordance with the present invention. An SPR chip prepared in accordance with example 2 was mounted in the instrument, modification of the chip to chelate nickel dication was effected in accordance with example 3, the modified chip surface was exposed to histidine-tagged Gal 11, and SPR response to introduction to the chip surface of SRB2 and SRB5 was investigated.

- Fig. 2 illustrates a sensorgram plotting resonance units as a function of time associated with this example. The flow rate of sample across the chip surface was 5 ul/min. The sensorgram of Fig. 2 is labeled with reference numerals that correspond to the steps of the experimental protocol below.
- 1. (t=50 s) chip description; 5% Ni ligand
 - 2. (t=390 s) end of injection of 1 mM NaOH
 - 3. (t=993 s) end of injection of 1% NiSO₄
 - 4. (t=1055 s) preinjection baseline
 - 5. (t=1555 s) end of injection of Gal 11 at 0.025 mg/ml
- 6. (t=1703 s) preinjection baseline
 - 7. (t=2222 s) end of injection of a second Gal 11 injection at the same concentration to achieve maximum chip coverage.
 - 8. (t=2343 s) preinjection baseline
 - 9. (t=2821 s) end of injection of SRB2 at 0.25 mg/ml
- 20 10. (t=2932 s) preinjection baseline
 - 11. (t--3438 s) end of injection of SRB2 at 0.5 mg/ml
 - 12. (t=3610 s) preinjection baseline
 - 13. (t=4116 s) end of injection of SRB5 at 0.25 mg/ml
 - 14. (t=4238 s) preinjection baseline
- 25 15. (t=4728 s) end of injection of SRB5 at 0.5 mg/ml
 - 16. (t=4842 s) preinjection baseline
 - 17. (t=5330 s) end of injection of myc-a at 0.25 mg/ml

Tabulated below are protein absorption response values (AbsResp; RU units) and response values relative to preceding baseline (RelResp; RU units) corresponding to the protocol steps above.

5

In contrast to the prior art embodiment examined in example 6, substantial binding of SRB2 to immobilized Gal 11 was observed. Importantly, no nonspecific binding of SRB5 to Gal 11 was observed. Specifically, 673 RUs of histidine-tagged Gal 11 bound to the surface exposing chelate coordinating nickel dication. The interaction of Gal 11 with SRB2 yielded an absorption of 1956 RUs at [SRB2] = 0.25mg/ml; at [SRB2] = 0.50 mg/ml, the absorption increased by an additional 176 RU's, for a total of 2132 RU's of bound protein. By contrast, at [SRB5] = 0.25 mg/ml the absorption was 47 RUs, while at [SRB5] = 0.5 mg/ml, the absorption was 73 RUs. Lastly, at [myc-a] = 0.25 mg/ml, the absorption was 450 RU's. These results clearly show that the biological molecule SRB2 bound to its binding partner Gal 11. The negative control SRB5 did not bind, and the positive control myc-a did bind.

As additional control experiments: (1) SRB2 was brought into contact with a chip incorporating a metal ion (prepared in accordance with example 3) that did not have

20

25

coordinated to it a the binding partner Gal 11, and (2) SRB5 was flowed over the chip after coordination of Gal 11 prior to the introduction of SRB2. No significant binding was observed in either case.

This experiment demonstrates that a biosensor surface prepared in accordance with the present invention, which provides biological binding partners oriented to stably expose recognition regions to an analyte solution, is superior to the prior art surface.

Example 8: Comparative Attempted SPR Determination of Monoclonal Antibodies Using Standard Dextran Chip Carrying T Cell Receptor

As in example 5, this example examines the sensitivity of the current state-of-the-art SPR chip, which comprises a layer of dextran on the gold SPR chip surface.

In this example, an attempt was made to study the interaction of a soluble 42 kD chimeric T cell receptor (TCR), herein referred to as ABC, with 2 monoclonal antibodies of MW = 155 kD (C1 and ßF1). Cl recognizes a conformational epitope thought to be near the active site of the receptor, while ßF1 recognizes a linear epitope of the constant domain. Although the structure of a TCR has not yet been solved, by analogy to IG structure, the C1 epitope should reside near the "top" of the protein and the ßF1 epitope near the "bottom", where the bottom is defined as the histidine tag of the protein. Probing the TCR with C1 demonstrated the availability of a defined site on the protein. The ratio of C1:ßF1 binding is the ratio of correctly:incorrectly folded protein, since C1 was shown to occlude the binding site of ßF1 when the TCR is bound to a surface (data not shown).

Fig. 3 illustrates a sensorgram plotting resonance units as a function of time associated with this example. The flow rate of sample across the chip surface was 5 ul/min. The sensorgram of Fig. 3 is labeled with reference numerals that correspond to the steps of the experimental protocol below.

- 1. (t=470 s) end of injection of EDC/NHS
- 2. (t=1044 s) end of injection of ABC at 0.125 mg/ml in PBS buffer
- 3. (t=1579 s) end of injection of ethanolamine
- 4. (t=2047 s) end of injection of C1 at 0.2 mg/ml in PBS
- 5. (t=2630 s) end of injection of BF1 at 0.2 mg/ml in PBS
- 6. (t=3152 s) end of injection of myc-a. at 0.2 mg/ml in PBS

30

Tabulated below are protein absorption response values (AbsResp; RU units) and response values relative to preceding baseline (RelResp; RU units) corresponding to the protocol steps above.

5		<u>Time</u>	AbsResp	RelResp
	1.	470.50	8717	
	2.	1044.00	25276	16558
	3.	1579.00	22216	13499
	4.	2047.00	22675	459
10	5.	2630.00	23728	1512
	6.	3152.00	23658	1442

13,499 RU's of ABC bound to the dextran surface. The interaction with Cl led to an absorption increase of 459 RUs, while that with ßF1 led to an additional 1053 RU's. Finally, the myc-a antibody did not bind. The ratio of C1:ABC was 1:29. The ratio of C1:B1 was 1:2.3. Since the molecular weight of C1 is 3.7 times that of ABC, the molar ratio of C1:ABC is 1:108, which implies that only 0.9% of the ABC molecules were recognized by C1.

This experiment demonstrates that the current state-of-the-art dextran chip does not provide the sensitivity necessary for determination of biological binding between ABC and C1.

Example 9: SPR Determination of Monoclonal Antibodies Using Chip Derivatized With Self-Assembled Monolayer of Alkyl Thiol Terminating In Metal Chelate Coordinated By Polyamino Acid-Tagged T Cell Receptor

As in examples 5 and 7, this example demonstrates the sensitivity of the SPR technique using a chip derivatized in accordance with the present invention. An SPR chip prepared in accordance with example 2 was mounted in the SPR instrument described above, modification of the chip to chelate nickel dication was effected in accordance with example 3, the modified chip surface was exposed to ABC (example 8) including a histidine tag at the constant domain, and SPR response to introduction to the chip surface of C1 and ßF1 was investigated.

Fig. 4 illustrates a sensorgram plotting resonance units as a function of time associated with this example. The flow rate of sample across the chip surface was 5 ul/min. The sensorgram of Fig. 4 is labeled with reference numerals that correspond to the steps of the experimental protocol below.

5

10

- 1. (t=42 s) chip description
- 2. (t=448 s) end of injection of 1 mM NAOH
- 3. (t=1017 s) end of injection of 1% NiSO₄
- 4. (t=1097 s) preinjection baseline
- 5. (t=1553 s) end of injection of ABC at 0.125 mg/ml; solution contains 0.02M imidazole
 - 6. (t=1696 s) preinjection baseline
 - 7. (t=2177 s) end of injection of C1 at 0.2 mg/ml; solution contains .02M imidazole
 - 8. (t=2288 s) preinjection baseline
- 9. (t=2781 s) end of injection of BF1 at .2 mg/ml; solution contains 0.02M imidazole
 - 10. (t=3075 s) preinjection baseline
- 11. (t=3604 s) end of injection of myc-a at 0.2 mg/ml; solution contains 0.02M imidazole

20

15

Tabulated below are protein absorption response values (AbsResp; RU units) and response values relative to preceding baseline (RelResp; RU units) corresponding to the protocol steps above.

	WO 98/31839		- 50 -	PCT/US98/01144
		<u>Time</u>	AbsResp	RelResp
	1.	42.00	7671	
	2.	448.50	7660	
	3.	1017.50	7730	
5	4.	1097.00	7695	
	5.	1553.50	9640	1945
	6.	1696.00	9634	1939
	7.	2177.00	10376	742
	8.	2288.00	10353	719
10	9.	2781.00	10761	408
	10.	3075.00	10739	386
	11.	3604.00	10830	91

1,945 RU's of ABC bound to the Ni surface. The interaction with C1 led to an absorption increase of 719 RU's, while that with ßF1 led to an additional 386 RU's. Finally, the myc-a antibody binding led to a 91 RU increase. The ratio of C1:ABC was 1:2.7. The ratio of C1:BF1 was 1.9:1. Since the molecular weight of C1 is 3.7 times that of ABC, the molar ratio of C1:ABC is 1:10, which implies that IO% of the ABC molecules were recognized by C1. In separate in vivo inhibition assays, we consistently found that 10% of the ABC was correctly folded. We therefore conclude that in this example 100% of the correctly folded ABC was oriented on the gold chip with the C1 binding site in the accessible "up" position with respect to the chip surface. This is in contrast to 9% retained activity in example 8.

This example demonstrates that essentially all of the binding partners immobilized at the chip surface in accordance with the present invention were oriented so as to expose in a direction away from the chip surface the recognition region of the binding partner for the biological molecule sought to be captured.

Example 10: Fabrication of Chip Derivatized With Self-Assembled Monolayer of Alkyl Thiol Terminating In Biological Binding Partner of DNA, To Expose DNA For Binding Studies

This prophetic example describes preparation of an alkyl thiol terminating at its

exposed end in a nucleic acid sequence, and a surface derivatized to carry a self-assembled monolayer including such species. Specifically, an SPR chip is primed to receive underderivatized dsDNA, provided by the user, which has been modified by the generation of an EcoR I site at one end.

A protected, single-stranded oligo is prepared via standard methods, as described in the Gene Assembler manual, Pharmacia Biosensor. The oligo is at least 25 base pairs long, and is a mixed, nonself-complementary sequence, terminated with a dG at the 3' end. The polymer-supported oligo is dried under vacuum overnight.

The 5' dimethoxytrityl (DMT) protecting group is deprotected at pH 3.0 in water for 10 minutes at room temperature. The reaction is stopped by freezing on dry ice, and the residue is filtered. See Ferentz, a.; Keating, T. a.; Verdine, G. L. <u>J. Am. Chem. Soc.</u> 115, 9006-9014 (1993)

From 11-mercaptoundec-1yl oligo(ethyleneglycol) (6; see example 2) is synthesized the S-DMT-protected derivative. See Zervas, L. L.; Photaki, I. <u>J.Am. Chem. Soc.</u> 84, 3887-3891 (1962). The 5' hydroxyl of the oligo is activated with carbonyl diimidazole. See Wachter, I.; Jabloski, J. a.; Ramachandran, K. L. <u>Nucleic Acids. Res.</u> 14, 7985-7994 (1986).

The protected alkyl thiol is added to the activated oligo in dioxane water for 30 minutes at room temperature. Excess reagent is washed away with dry dioxane, followed by methanol, and the product is dried in a desiccator overnight. The remaining protecting groups and the solid support are deprotected by treating with 25% aqueous ammonia for 6 hours at 60° C. The aqueous ammonia is chilled at O° for 30 minutes, the polymer support is filtered, and the ammonia evaporated.

The derivatized oligo is purified via 8% PAGE-7M urea, then via G-10 Sephadex chromatography. The oligo is reduced with silver nitrate/DTT and rechromatographed. See Connolly, B. a.; Rider, P. Nucleic Acids Res. 12, 4485-4502 (1985)

An oligo of sequence complementary to that made in step 1, with an additional 4 bp's at the 5' end (3'-TTAA-5') is purchased. The purified, derivatized oligo from the previous step is hybridized with the complementary oligo by denaturation at 75° C, and annealed at 55° C. This yields an alkyl thiol attached to a dsDNA, which is terminated with an EcoR I restriction enzyme site. EcoR I is widely available commercially, for example from New England Biolabs.

RECTIFIED SHEET (RULE 91) ISA/EP

5

10

This DNA ligand is mixed with a self-assembled monolayer-forming, nonspecific binding-inhibiting species, specifically, 11-mercaptoundec-1yl oligo(ethyleneglycol) (6; see example 2) in a 5:95 molar ratio, respectively. The chip surface is prepared as described in example 2. This completes the preparation of a commercial chip.

Example 11: Modification of Chip Derivatized With Self-Assembled Monolayer of Alkyl Thiol Terminating In Biological Binding Partner of DNA, To Expose DNA For Binding Studies

In this prophetic example, selected dsDNA that the user wishes to present on the chip surface is cut, via standard methods, with EcoR I. The cut DNA is ligated to the DNA already on the chip using DNA ligase (New England Biolabs). The chip is now ready for use in a biosensor.

Advantages of the chip prepared in accordance with examples 10 and 11 follow. The current state-of-the-art chip, available from Pharmacia Biosensor for use in the BIAcoreTM SPR instrument, presents a dextran surface to which strepavidin is attached. The user must either derivative the desired DNA with biotin, or purchase biotinylated DNA. In the former case, the reaction is difficult and biotinylation may occur at any number of sites, leading to a lack of control over orientation on the chip. Furthermore, biotinylation may block an interaction site on the DNA. Commercially available biotinylated DNA is very expensive and cannot be obtained in lengths much greater than 70 base pairs. The derivatized DNA then is attached to the chip surface via interaction between the biotin and the strepavidin.

Another disadvantage of the prior art strepavidin/biotin technique includes the fact that many of the compounds which bind negatively charged DNA are positively charged. Since the dextran surface retains a number of negatively charged carboxylates, interactions of an electrostatic nature may be mistaken for binding with the target DNA. Also, kinetic analyses of binding would be compromised due to the additional attractive force created by the negative carboxylates. Still another disadvantage of the prior art technique is that the strepavidin on the chip surface gives rise to nonspecific interactions with many target compounds.

The procedure described in this example for the preparation of the DNA chip may be applied to the preparation of an analogous RNA chip.

30

5

Example 12: Fabrication and Modification of Chip Derivatized With Self-Assembled

Monolayer of Alkyl Thiol Terminating In Biological Binding Partner of DNA, To Expose

DNA For Binding Studies

This example describes preparation of a SAM terminating at its exposed end in a nucleic acid sequence, and a surface derivatized to carry a self-assembled monolayer including this species in combination with a background layer of inert SAM-forming species. In particular, a SAM presenting short strands of single-stranded DNA (ssDNA; 34, with reference to Figs. 8-10) above a background layer of inert non-binding thiols 30 was prepared, followed by hybridization of double-stranded DNA containing specific protein binding sites to the chip surface, the double-stranded DNA having a single stranded tail complementary to that presented by the chip. Specifically, an SPR chip was primed to receive underderivatized dsDNA modified by the generation of an EcoR I site at one end.

An SAM-forming nucleic acid species 34 was synthesized as follows. Species (1) was reacted with tosyl chloride in pyridine at room temperature for 4-6 hours to give species (8):

(8)
$$H_2C=CH-(CH_2)_9-(OCH_2CH_2)_3-OS(0)_2(C_6H_4)CH_3$$

This was reacted with NaN₃ in N,N-dimethylformamide at 60°C for approximately 4-8 hours giving species (9):

(9)
$$H_2C=CH-(CH_2)_0-(OCH_2CH_2)_3-N=N=N$$

Species (9) was reacted according to one of two methods. In the first method, species (9) was reacted with PPh₃ in THF followed by reaction with water. In a preferred method, reaction took place with LiAlH₄, refluxing for two hours in THF, followed by reaction with water. In either case species (10) was recovered:

30 (10)
$$H_2C=CH-(CH_2)_0-(OCH_2CH_2)_3NH_2$$

WO 98/31839 - 54 - PCT/US98/01144

Species (10) was reacted with (CH₃)₃C-O-C(O)-O-C(O)-O-C(CH₃)₃ and Et₃N in DMF for 0.5 hours at 50°C giving species (11):

(11)
$$II_{2}C=CH-(CH_{2})_{9}-(OCH_{2}CH_{2})_{3}N(H)C(O)-O-C(CH_{3})_{3}$$

which was reacted with six equivalents of $CH_3C(O)SH$ and $CH_3-C(CH_3)(CN)-N = N-C(CH_3)(CN)-CH_3$. The reaction mixture was irradiated with a medium pressure 450 Watt lamp (Hanovia) for five hours giving species (12):

10 (12)
$$(H_3C)C(O)S(CH_2)_{11}-(OCH_2CH_2)_3N(H)C(O)-O-C(CH_3)_3$$

Species (12) was reacted with HCl/MeOH(0.1M) and refluxed for 4-6 hours to give species (13):

15 (13)
$$HS(CH_2)_{11}$$
-(OCH₂CH₂)₃NH₂

which was reacted with I_2 , O_2 , and $CH_3(CH_2)_3SH$ in dimethoxyethane for 4 hours to give species (14):

20 (14)
$$[S(CH_2)_{11}-(OCH_2CH_2)_3NH_2]_2$$

A 10-base strand of DNA was synthesized on a DNA synthesizer. While still attached to the resin, the 5'-deprotected DNA was reacted with carbonyldiimidazole (CDI) to give the 5'-imidazolylacylated product. This was subsequently reacted with species (14), first in dioxane for 0.5-1 hour, then with NH₃ at 60°C for 6-8 hours to give species (15):

(15)
$$[S(CH2)11-(OCH2CH2)3N(H)C(O)-O-DNA-OH(3')]2$$

Species (15) was removed from the resin by standard purification techniques except that NH₄OH at 65°C for 8 hrs was used, giving (16):

(16) [S(CH₂)₁₁-(OCH₂CH₂)₃N(H)C(O)-O-DNA]₂

The product, after removal from resin after purification (HPLC), was a thiol derivatized with single-stranded DNA. HPLC analysis showed the generation of a new species (50-75% pure) that eluted from the column upon purification much later than the underivatized 10-mer DNA and slightly slower than the 10-mer DNA with the trityl group still attached to the 5' end. The elution profile of the product is consistent with expected results for DNA derivatized with the alkyl thiol chain.

Species (16) formed pairs of exposed nucleic acid strands, with the -S-S-bridge in the center of the molecule adhering to the surface. Species (16) is represented as species 34 in the figures.

The selected dsDNA desirably immobilized at the surface was then cut, via standard methods, with EcoR I. The cut DNA was ligated to the DNA already on the chip using DNA ligase, resulting in a chip ready for use in a biosensor.

To form a self-assembled monolayer, SAM-forming ssDNA species (16, 34 in the figures) was mixed with the inert, non-binding ethyleneglycol-terminated thiol (6; 30 in the figures) in mM ethanol solution in a molar percent of inert species of about 0.5-3%. A gold 26-coated glass substrate 22 then was incubated in this solution. A SAM 28 was formed on the gold surface 24. It was assumed that any DNA not derivatized with thiol did not bind the gold surface. Additionally, any amino-thiol that did not react with the 5' hydroxyl of the DNA would have been lost during the extensive washing steps while the DNA was still resin-bound. Therefore, DNA-SAMs were formed without further purification. Good SAM formation from acetonitrile was significantly more effective than from ethanol, forming (per SPR analysis) ordered SAM arrays when the gold substrate was incubated at 45°C for 12 hours. The surfaces were well behaved in that they resisted the binding of proteins and (as Figs. 5-7 and related discussion show) these surfaces hybridized DNA if and only if the DNA had a single stranded tail of exactly complementary sequence to that presented by the chip.

Once dsDNA 36 was hybridized to the covalently-immobilized single-stranded

DNA 34 at the surface, the nick 40 in the coding strand was covalently joined by DNA ligase but only in cases in which the 5' hydroxyl of the incoming oligo was phosphorylated. It was observed that performing a DNA ligation reaction resulted in more

10

15

20

DNA stably bound to the surface but only if the 5' hydroxyl was first phosphorylated. When DNA containing Gal4 recognition sites was hybridized to the DNA-SAM, it selectively bound Gal4 protein but not another DNA-binding protein Lex-B17. Our results indicate that we have generated a DNA-presenting SAM.

Example 13: Characterization of Chip Derivatized With Self-Assembled Monolayer of Alkyl Thiol Terminating In Biological Binding Partner of DNA

Varying amounts of the ssDNA-derivatized disulfide (16) were mixed with the inert, tri-ethylene glycol terminated thiol (6) which defined the major component (1mM) in acetonitrile solutions. Pre-cleaned (H₂SO₄/H₂O), gold-coated glass slides were incubated at 45°C for 8-12h in the solutions. The slides were cut and mounted on plastic CM-5 SPR chip cassettes (Pharmacia). The experimental chips were docked in a BIΛCoreTM SPR instrument and experiments were performed to assay the ability of the chip to hybridize single-stranded DNA (ssDNA) complementary to the strand of the DNA-derivatized thiol.

Three DNA samples were sequentially injected over the same flow cell of a chip. The samples contained double-stranded DNA (dsDNA) containing 2 Gal4 protein binding sites and the three DNA samples included: 1) DNA without a single-stranded tail, 2) DNA with a 10-base single stranded tail whose sequence content was complementary to strand 34 presented by the chip, but having a scrambled sequence and 3) DNA with a 10-base single stranded tail exactly complementary to that presented by the chip. 35mL of each DNA sample (DNA concentration=[14pM/mL] in 400mM NaCl) as separately injected over the DNA-SAM. Experiments were run at RT at a constant flow rate of 5mL/min in PBS (137mM NaCl). As discussed above, the binding of molecules to a chip surface is detected as a net increase in resonance units (RUs). A net change in RUs resulted only after the injection of sample (3), that is, the DNA bearing a single-stranded tail whose sequence was exactly complementary to strand 34 of the DNA-thiol SAM immobilized on the chip.

Fig. 5 illustrates a sensorgram plotting RU as a function of time associated with the experiments involving DNA with a single-stranded scrambled tail and DNA with a 10-base single stranded tail complementary to nucleic acid strand 34 presented by the chip. The sensorgram of Fig. 5 is labeled with reference numerals that correspond to the steps of the experimental protocol below.

5

5

- 1. (t=130 sec) end of injection of 137mM NaCl buffer.
- 2. (t=205 sec) preinjection baseline.
- 3. (t=519 sec) end of injection of DNA with 10-base single-stranded scrambled "tail".
- 4. (t=624 sec) preinjection baseline.
 - 5. (t=1003 sec) end of injection of DNA with 10-base single-stranded tail complementary to that presented by the chip.

Tabulated below are DNA absorption response values (AbsResp; RU units) and response values relative to preceding baseline (RelResp; RU units) corresponding to the protocol steps above.

		<u>Time</u>	<u>AbsResp</u>	RelResp
	1.	130.50	11726	-4
15	2.	206.50	11722	-7
	3.	519.50	11719	-3
	4.	624.50	11710	-8
	5.	1003.00	11822	112

Fig. 6 is a sensorgram plotting RU as a function of time associated with injection of DNA without a single-stranded tail. The sensorgram of Fig. 6 is labeled with reference numerals that correspond to the steps of the experimental protocol below.

- 1. (t=259 sec) chip description
- 2. (t=787 sec) end of injection of DNA without tail

Tabulated below are DNA absorption response values and response values relative to preceding baseline corresponding to the protocol steps above.

30		<u>Time</u>	<u>AbsResp</u>	<u>RelResp</u>
	1.	259.00	12380	0
	2.	787.00	12383	2

This experiment demonstrates that a biosensor surface prepared in accordance with the invention provides a nucleotide sequence oriented to expose away from the chip surface the nucleotide 34 for binding to a complementary nucleotide.

We found that the DNA hybridized to the chip was more stably bound if it was enzymatically ligated to the DNA presented by the chip. To do this, the chips were docked in the BIAcoreTM SPR instrument and equilibrated in PBS. Baseline measurements were recorded for each flow cell. The chips were then removed from the instrument and were bathed in 100mL of a solution containing DNA with a 10-base single-stranded tail complementary to strand 34 presented by the chip for 0.5h at RT. The excess solution was removed from the chip surface and 200mL of DNA ligase in ligase buffer was added to mend nick 40 in the strand. The stability of the bound DNA increased if and only if DNA ligase was present and the 5' end of the incoming synthetic DNA strand was phosphorylated. These results reflect the advantage of using DNA ligase to join two DNA strands through a 5' phosphate group.

15

Example 14: SPR Determination of Specific Protein Binding to DNA Chip

Example 13 could specifically bind proteins to binding sites on the hybridized DNA while resisting the non-specific adsorption of irrelevant proteins. In particular, SPR experiments were conducted to investigate the binding of Gal4(1-100) as opposed to Lex-B17 on chips carrying hybridized DNA bearing 2Gal4 protein binding sites. Fig. 7 is a sensorgram plotting resonance unit as a function of time associated with this example. The sensorgram of Fig. 7 is labled with reference numerals that correspond to the steps of the experimental protocol below.



WO 98/31839 - 59 - PCT/US98/01144

- 1. (t=77 sec) chip description
- 2. (t=335 sec) end of injection of 137 mM NaCl
- 3. (t=677 sec) end of injection of DNA including 10-base pair complementary to that presented by the chip
 - 4. (t=923 sec) preinjection baseline

5

- 5. (t=1275 sec) end of injection of Lex-B17 (0.125 mg/ml)
- 6. (t=1327 sec) preinjection baseline
- 7. (t=1700 sec) end of injection of Gal4 (0.125 mg/ml)

Tabulated below are protein absorption response values and response values relative to preceding baseline corresponding to the protocol steps above.

		<u>Time</u>	$\Delta bsResp$	<u>RelResp</u>
	1.	77.50	10651	0
15	2.	335.50	10658	7
	3.	677.50	10791	133
	4.	923.00	10732	-59
	5.	1275.50	10762	29
	6.	1327.50	10741	-20
20	7.	1700.00	10955	213

Experiments were performed also to determine whether the concentration of the SAM-forming nucleotide on the chip had an effect on protein binding. Table 1 shows a comparison of four different chips with different DNA concentration at the chip surface,

25 the DNA including 2 Gal4 protein binding sites.

Table 1.

dsDNA CONTAINING 2 GAL4 BINDING SITES (D RUs)	GAL4 BOUND (RUs)	LEX B17 BOUND (RUs)
52	164	
74	213	0
441	365	
1378	1079	96

10

15

20

5

SPR experiments showed that hybridized DNA bearing 2 Gal4 protein binding sites preferrentially bound Gal4(1-100) protein over Lex-B17. Additionally, there appears to be a direct correlation between the amount of DNA hybridized to the chip and the amount of Gal4 that subsequently bound to it.

The observed preference of chip-hybridized DNA containing Gal4 binding sites for Gal4 protein over Lex-B17 was reversed when DNA bearing Lex binding sites was hybridized to the chip surface (see Table 2). DNA containing 2 Lex binding sites and a single-stranded complementary tail was annealed, then ligated to a ssDNA chip. The chip was then docked in a BIAcoreTM SPR instrument and equimolar concentrations of either Lex-B17 or Gal4 were separately passed over three flow cells of the same chip.

Table 2.

FLOW CELL	GAL4 BOUND (RUs)	LEX B17 BOUND(RUs)
· I		363
2	136	
3		345

25

Example 15: Detection of Small-Molecule Interactions Based on Electron Transfer

This prophetic example describes biospecific SAMs (S-R-ch or X-R-NA SAMs) that are

part of a biosensor for the detection of protein-protein, protein-small molecule, protein-DNA, or DNA-DNA (hybridization) interactions based on electron transfer. A biosensor for detecting DNA-DNA interactions is described first, with reference to the figures. A SAM comprised of DNA-thiol 34 (described in Examples 12 and 13) as the minor component and a tri-ethyleneglycol terminated alkane thiol ((6); 30 in the figures) as the major component in a ratio of about 3:97 is formed on a gold-coated electrode. With reference to Fig. 9, double stranded DNA (dsDNA) 36 having a single stranded tail 38, complementary to that of the DNA-thiol 34, is hybridized to the DNA-SAM on the electrode. The nick 40 between the 3' end of DNA-thiol 34 and the 5' end of incoming dsDNA 36 is covalently mended with the enzyme DNA ligase. The result is that the DNA of the DNA-SAM has been covalently extended with DNA of interest (dsDNA 42). Incoming dsDNA 42 contains a polylinker (a stretch of DNA containing recognition sequences at which specific restriction enzymes cleave dsDNA) so that it can be cut with restriction enzyme 46, and then E. coli produced DNA 50 (Fig. 10), cut with enzyme 46, is ligated onto the SAM to form surface-immobilized dsDNA 52. The nick in the coding strand optionally is mended. Since only the sense strand 54 is covalently attached to the SAM, heat or chemical treatment will dissociate the anti-sense strand 56.

After dissociation, the resultant single-stranded DNA (ssDNA 72, now with reference to Figs. 11 and 12) then is probed, by hybridization, with specific DNA sequences 70. If specific DNA sequences 70 are the complement of sequences 72, then hybridization will occur and a flow of current will be measured at ammeter 74 that is distinguishable from situations in which hybridization does not occur.

Referring now to Fig. 13, where different ssDNA strands are provided in each of the different isolated regions 84, 86, 88, 100... when potential difference is placed across the two electrodes, distinguishable conductance occurs only when the two DNA species by bridize to complete the circuit, thus existence of unknown samples can be determined.

The array schematically illustrated in Fig. 13 is advantageous in that either or both of the SAMs on electrodes 62 or 66 can be reused. For example, a series of different species provided on electrode 66 can be used repeatedly with a series of different electrodes 62 each including a single, but different species across the entire electrode. Similarly, a single electrode 62 can be used with a variety of different arrays on different electrodes 66.

30

10



WO 98/31839 - 62 - PCT/US98/01144

This assay is useful in sequencing the human genome. To determine what DNA sequence abuts the last sequence mapped, random sequences of DNA from a certain chromosome are obtained by standard PCR techniques. By using PCR primers that contain the recognition sequence of a particular restriction enzyme, all the DNA samples, generated from the parent DNA, have identical flanking restriction enzyme sites. As a heterologous species, they are ligated into a bacterial expression vector which is used to transform E.coli cells. Bacterial colonies, each expressing a single DNA species, are picked and the plasmid DNA is extracted. Of the hundreds of thousands of DNA species. only oligos that also contain part of the previously-identified abutting sequence need to be identified. The anti-sense strand first is "probed" or the abutting sequence hybridized to the unknown DNA oligos and then only the oligos that hybridize to the probe need to be sequenced. Each E. coli derived DNA species actually must be separately immobilized to facilitate characterization once an interaction has been detected. This is accomplished when arrays of micro-electrodes or AFM tips, each carrying a single DNA species, interfaces with arrays of probe-ocaring micro-electrodes. Photo-resist methods or other methods described above are employed to create a uniform array of gold islands bordered by hydrophobic surfaces. The DNA-SAM is uniformly assembled over the array and polylinker attached. Attachment of multiple DNA species is carried out by laying down individual droplets of single species DNA and ligase containing solution over the spatially addressable gold islands in the array. Alternatively, a microelectrode array displaying a single species interfaces with a diverse DNA probe array synthesized by light-directed spatially addressable synthesis (Fodor, S.P.A., Read, J.L. Pirrung, M.C., Stryer, L., Lu, A.T., Solas, D., "Light-Directed, Spatially Addressable Parallel Chemical Synthesis", Science, 251 (1991) pp. 767-773). Thus a generic DNA-SAM is used to present specific oligos of any length or sequence for interaction studies with other molecules. Similar 25 strategies enable the detection of protein-peptide interactions by immobilizing histidinetagged proteins to one array of NTA-SAM derivatized microelectrodes and interfacing it with spatially addressable peptides immobilized on a second array.

0 Example 16: DNA Computer

In this prophetic example, a DNA computer is made (Adleman, L.M., "Molecular Computation of Solutions to Combinatorial Problems", *Science*, **266** (1994) pp. 1021).

The four bases that make up DNA are used to code information sequentially along the molecule. Like the binary code in electronic computers, it can be used to define problems, perform sequential processing (a program) and read out solutions. DNA computing requires that random DNA (all possible solutions) be probed with short oligos (certain criteria that must be met). The solution is the sequence of the random DNA that satisfies all the criteria. To solve the problem of how to identify which of the random oligos satisfies all the criteria (exactly hybridized to all the probe oligos), a DNA-SAM derivatized with random ssDNA is probed with an array of probe (starting point) DNA. Specific oligos that code for certain criteria are added into the solution. The addition of DNA ligase makes the sequence of the "solution" strand permanent. Which probe now carries the exact sequence is detected making use of the time dependence of electron transfer through double stranded DNA compared with transfer through single stranded DNA, as described above. The probe sequence that is shown to carry the solution is ascertained by PCRing the species of that single probe.

15

20

Example 17 - Electronic-Property Determination of Disease State Involving Mis-Folded Protein

This prophetic example describes a simple technique for determining a disease state involving mis-folded protein in an early stage of the disease.

With reference to Fig. 16, a system 120 is provided in which normal peptides are presented by SAM 124. The normal peptide is directly attached to a thiol and then the derivatized species used to form a mixed SAM (with NSB-resistant species 30) or the peptide is attached to a metal clate via polyamino acid tag. In a particularly sensitive arrangement, the peptide is attached to the surface of electrode 122 via a "Molecular Wire" (*Science*, 271, 1705-1707, 1996, incorporated herein by reference).

Fluid or tissue extracts from a mammal suspected to be infected with the mis-folded protein disease state such as TSE are injected into inlet 93 of container 94. Where prions are present, they cause a conformational change in the surface-immobilized normal protein, which changes the electronic properties of the capacitor system, as detected at impedance sensor 99.

The normal protein need not be immobilized at the surface but may be free in the capacitor medium.

The medium of the system may contain other elements such as catalysts, sugars,

lipids, sulfated glycosaminoglycans or solvents to hasten fibril and plaque formation which occurs in the diseased state. It may also contain normal protein to amplify the detection of the disease state when prion is present the added normal protein will become mis-folded and, in turn, cause the surface-immobilized protein to become mis-folded. Other physical parameters of the systems, such as temperature, pressure, electrical or magnetic field densities can be optimized to favor more rapid progression of conformational changes. For example, the system can be pulsed with various frequencies of electromagnetic radiation (e.g., Ghz range) for this purpose.

The advantages of the system over prior art antibody staining of infected tissue sections include the following, infectious samples are in solution and not dependent upon a level of prion aggregation before being detectable and so earlier detection of infection is possible. The method of detection does not depend upon the viability of other less robust proteins such as antibodies, therefore the system can be subjected to harsh physical or chemical conditions (organic solvents such as toluene, THF, ACN, phenol and the like, and pH outside of the physiological range of 6.8-8.2) or frequencies which can promote the conformational changes that characterize the disease. The system of the invention can be a more sensitive assay because single molecule conformational changes are detected. When MHZ frequencies are used in the detection, a 1% change in the conformation of the sample volume can be detected (six significant figures). Greater variety of samples and smaller sample sizes (needle biopsies, blood, saliva, urine, cerebrospinal or ocular fluid, lymphoid tissues, etc.) can be used. The method is specific because it measures the very change that determines the disease state, that is, the conformational change. The method is inexpensive and easily scalable, lending itself to mass screenings. The variety of sample types compatible with the method of detection makes the system applicable to the detection of prions in live animals, food products derived from animals, and animal feed. This allows of prevention of the spread of disease at several stages. Interpretation of test results does not require a high level of skill so unskilled personnel can administer the test.

Those skilled in the art would readily appreciate that all parameters listed herein are meant to be exemplary and actual parameters will depend upon the specific application for which the methods and apparatus of the present invention are being used. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention

may be practiced otherwise than as specifically described.

What is claimed is:

CLAIMS

1. A method comprising:

introducing an electron into a first biological species that is one of a pair of biological binding partners defined by the first biological species and a second biological species;

passing the electron through the first species and into the second species; and removing the electron from the second species.

- 2. A method as in claim 1, wherein the first biological species is a first nucleic acid strand and the second biological species is a second nucleic acid strand, the method comprising introducing an electron into a first nucleic acid strand, passing the electron into a second nucleic acid strand that is the complement of the first nucleic acid strand, and removing the electron from the second nucleic acid strand.
- 15 3. A method as in claim 1, wherein the second biological binding partner is immobilized at a surface of an article, and the method involves removing the electron from the second species by passing the electron into the article.
- A method as in claim 3, wherein the second biological species is immobilized to a
 self-assembled monolayer-forming species that forms part of a self-assembled monolayer at the surface.
 - 5. A method as in claim 3, wherein the first biological species is immobilized at a surface of an article different from the article at which the second biological species is immobilized, the method involving introducing an electron into the first biological species from the article at which it is immobilized, and passing the electron from the second biological species into the article at which it is immobilized.
 - 6. A method as in claim 1, wherein the first biological species is immobilized via a self-assembled monolayer-forming species on a surface of a first article and the second biological species is immobilized via a self-assembled monolayer-forming species at a surface of a second article, the method involving passing an electron from the first article,

through the self-assembled monolayer-forming species into the first biological species and removing the electron from the second biological species, through the self-assembled monolayer-forming species at which it is immobilized, into the second article.

- 5 7. A method as in claim 6, wherein the first biological species and second biological species each are nucleic acid strands.
 - 8. A method as in claim 7, wherein the first and second biological species each are nucleic acid strands covalently coupled to self-assembled monolayer-forming species.

9. A method as in claim 8, wherein the first biological species is immobilized to the surface of the first article via a self-assembled monlayer-forming species that is part of a self-assembled monolayer including other, similar first biological species, wherein at least

90% of the first biological species are isolated from all other first biological species.

- 10. A method as in claim 9, wherein the first biological species are isolated from each other by a biologically-inert, self-assembled monolayer-forming species.
- 11. A method as in claim 4, wherein the first biological species is immobilized at the surface of the first article via a self-assembled monolayer-forming species, and the surface of the first article includes a plurality of first biological species wherein at least 90% of the first biological species are isolated from all other first biological species.
- 12. A method as in claim 11, wherein the first biological species are isolated from each other by a biologically-inert, self-assembled monolayer-forming species.
 - 13. A method as in claim 1, wherein the second biological species is immobilized at a surface of an article via a self-assembled monolayer-forming species X-R-Ch-M, where X, R, Ch, and M each are selected such that X represents a functional group that adheres to the surface. R represents a spacer moiety that promotes formation of a self-assembled monolayer of the species. Ch represents a chelating agent that coordinates a metal ion, and M represents a metal ion, wherein the first biological species coordinates the metal ion.

- 14. A method as in claim 13, wherein the first biological species is immobilized at a surface on which is immobilized a plurality of biological species wherein at least 90% of the biological species are isolated from all other biological species.
- 5 15. A method as in claim 14, wherein at least 90% of the first biological species at the surface are separated from each other by at least 5 nm.
 - 16. A method as in claim 15, wherein the first biological species each are immobilized at the surface of the first article via self-assembled monolayer-forming species and are isolated from each other by biologically-inert self-assembled monolayer-forming species.
 - 17. A method as in claim 16, wherein the ratio of biologically-inert self-assembled monolayer-forming species to self-assembled monolayer-forming species via which the biological species are immobilized is at least 70:30.

18. A method as in claim 17, wherein the article at which the first biological species is immobilized and the article at which the second biological species is immobilized each are electrodes connected via an electrical circuit that measures electron transfer through the first and second biological species.

20

19. A method as in claim 1, wherein the article at which the first biological species is immobilized and the article at which the second biological species is immobilized each are electrodes connected via an electrical circuit that measures electron transfer through the first and second biological species.

- 20. A method comprising passing an electron through a biological species linked to a self-assembled monolayer-forming species immobilized at a surface of an article.
- 21. A method comprising:
- providing a first biological species that is linked to a surface of a first article via a self-assembled monolayer and a second biological species that is linked to the surface via a self-assembled monolayer; and

passing an electron through the first biological species while the second biological species does not pass an electron.

22. A method as in claim 21, comprising passing an electron through the first biological species by causing a biological binding partner of the first biological species to bind to the first biological species and introducing an electron from the binding partner into the first biological species, passing the electron through the first biological species, and passing the electron through a self-assembled monolayer-forming species to which the first biological species is immobilized at the surface into the article.

- 23. A method as in claim 22, wherein the first biological species is a nucleic acid strand and the biological binding partner of the first biological species is a second nucleic acid strand complementary to the first nucleic acid strand.
- 15 24. A method as in claim 21, wherein the first and second biological species each are nucleic acid strands.
 - 25. A method as in claim 21, wherein the first biological species and second biological species are members of an array of a plurality of similar biological species with binding properties distinct from each other, the method involving positioning a surface of a second article, carrying a self-assembled monolayer at which is immobilized a biological binding partner of the first biological species, in proximity of the surface of the first article, allowing the biological binding partner to biologically bind to the first biological species and to be positioned in proximity of the second biological species such that biological binding could occur if the binding partner were specific for the second biological species.
 15 binding could occur if the binding partner were specific for the second biological species.
 16 applying an electrical potential to the biological binding partner, and allowing an electron to pass from the biological binding partner to the first biological species but not to the second biological species.
 - 30 26. A method as in claim 25, comprising determining electron transfer from the biological binding partner to the first biological species while observing a lack of electron transfer from the biological binding partner to the second biological species.

27. A method comprising:

providing a first biological species immobilized at a surface of a first article and a second biological species immobilized to the surface of the first article; and

passing an electron through the first biological species while the second biological species does not pass an electron.

- 28. An article comprising a first electrode having a surface, a second electrode having a surface, an electrical circuit connecting the first and second electrodes, a first biological species immobilized at a surface of the first electrode, and a biological binding partner of the first biological species immobilized at the second electrode.
- 29. An article as in claim 28, wherein the first biological species is immobilized at the surface of the first electrode via a self-assembled monolayer.
- 15 30. A method as in claim 29, wherein the biological binding paraner of the first biological species is immobilized, at the surface of the second electrode, via a self-assembled monolayer.
- 31. A method as in claim 28, further comprising a second biological species immobilized at the surface of the first electrode, the second biological species not being specific for the biological binding partner of the first biological species.
 - 32. An article as in claim 31, wherein the first biological species and second biological species are isolated from each other.
 - 33. An article as in claim 32, wherein the first and second biological species each are immobilized at the surface of the first electrode via a self-assembled monolayer, and are isolated from each other by a biologically-inert self-assembled monolayer-forming species.
- 30 34. A method as in claim 33, wherein the first and second biological species are separated from each other by at least 5 nm.

35. An article as in claim 34, wherein the surface of the first electrode carries a self-assembled monolayer made up of self-assembled monolayer-forming species linked to biological species and biologically-inert self-assembled monolayer-forming species in a ratio of less than or equal to 30:70.

5

36. A method comprising:

determining the interaction of a first molecule with a second molecule by detecting a change in electronic configuration of the second molecule due to interaction of the second molecule with the first molecule.

10

- 37. A method as in claim 36, the determining step comprising exposing the second molecule to a medium suspected of containing the first molecule and determining the presence of the first molecule in the medium.
- 15 38. A method as in claim 36, wherein the second molecule is immobilized at a surface via a spacer moiety and the determining step comprises allowing the first molecule to interact with the second molecule and determining a change in electronic configuration in the second molecule via detection involving electron transfer through the spacer moiety between the surface and the second molecule.

20

39. A method as in claim 36, the determining step comprising detecting a change in impedence of a system including the second molecule, after interaction with the first molecule, relative to an impedence measurement of the system without interaction with the first molecule.

25

- 40. A method comprising:
 - immobilizing a protein at a surface; inducing a change in the conformation of the protein at the surface; and

detecting the change in conformation.

30

41. A method as in claim 40, the determining step involving measuring a change in an electrical property associated with the proteins.

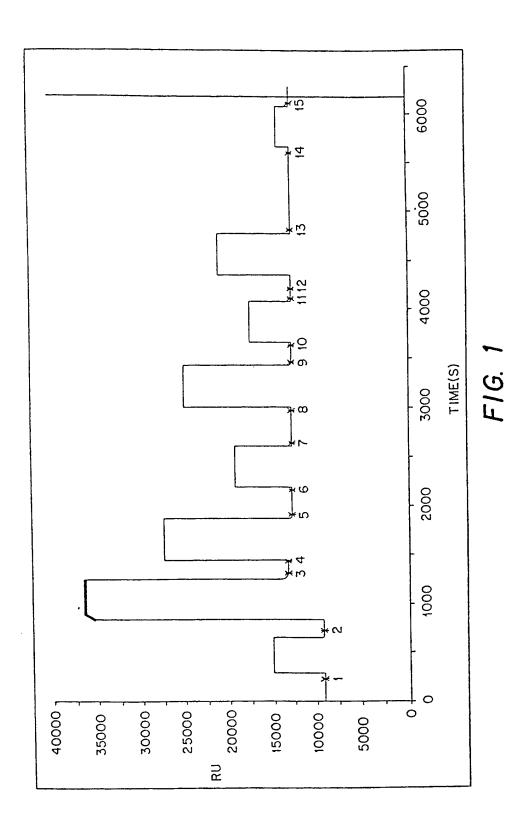
- 42. A method as in claim 40, wherein the inducing step involves exposing the protein to a prion.
- 43. A method comprising:
- 5 providing a protein;
 exposing the protein to a prion or associated cofactor; and
 determining a change in conformation of the protein induced by exposure of the
 protein to the prion.
- 10 44. A method as in claim 43, wherein the exposing step involves exposing a physiological sample of a living mammal to the protein.
 - 45. A method as in claim 43, wherein the exposing step involving exposing the protein to the prion in the proximity of medical diagnostic apparatus.
 - 46. A method as in claim 45, wherein the exposing step involves exposing the protein to the prion in proximity to an electrode surface constructed and arranged for determination of a change in electric property of the protein upon mis-folding.
- 47. A method as in claim 46, wherein the exposing step involves exposing the protein, immobilized at a surface of an electrode, to the prion.
- 48. A method comprising:

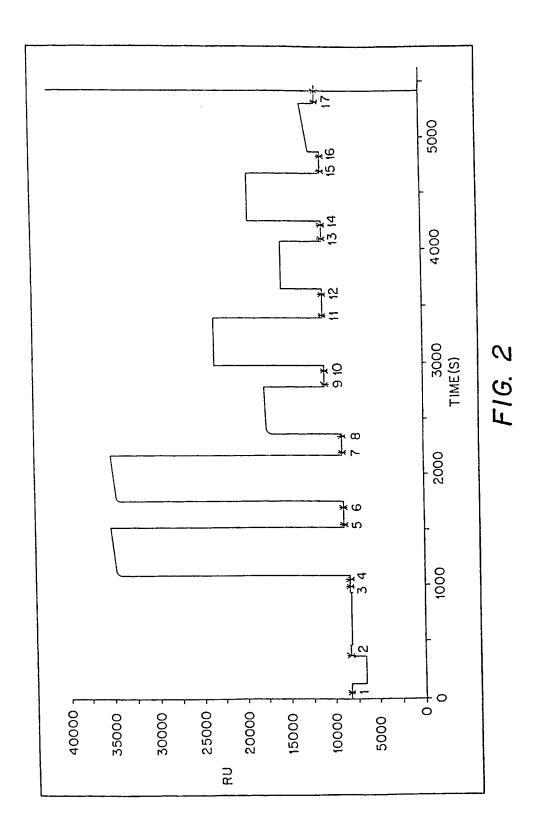
 determining a disease state involving mis-folded protein in a physiological sample
 of a living mammal.
 - 49. A method as in claim 48, the determining step involving exposing normal protein to the physiological sample and determining mis-folding in the normal protein.
- 30 50. A method as in claim 49, involving exposing normal protein, immobilized at a surface of an electrode, to the physiological sample; and determining an change in electronic property of the normal protein immobilized at

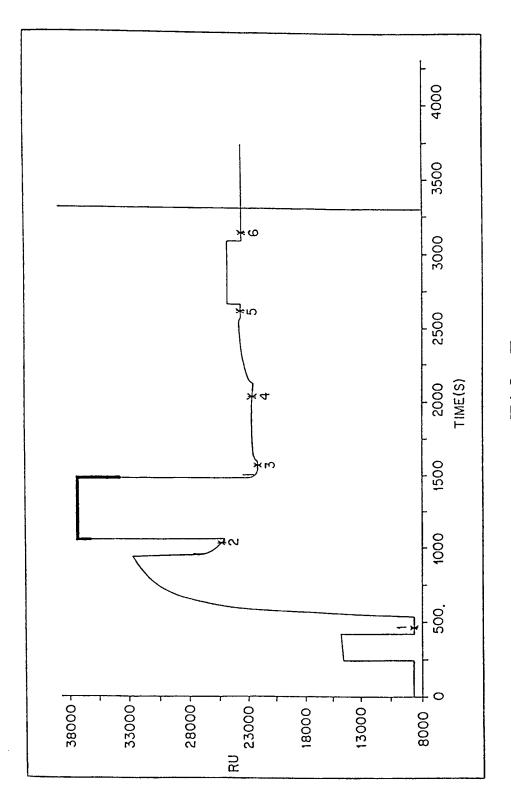
the surface of the electrode.

5

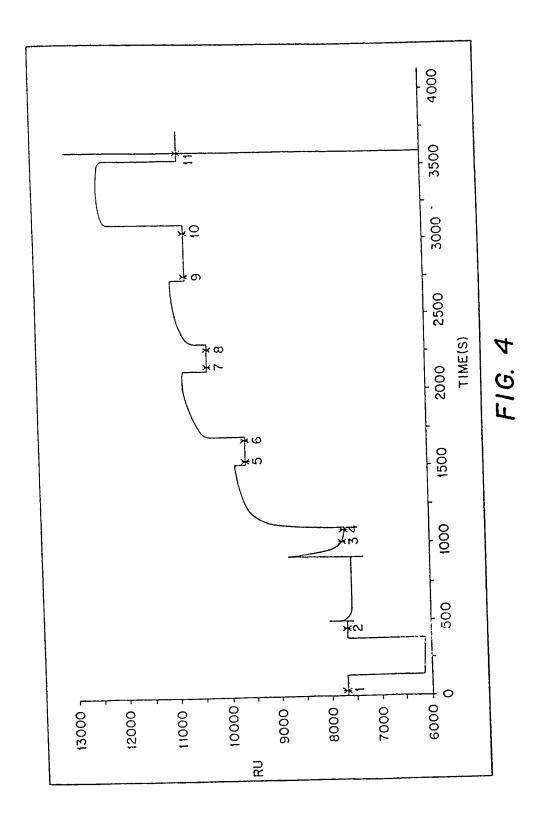
51. A method as in claim 50, wherein the electrode forms part of a impedance detection system.

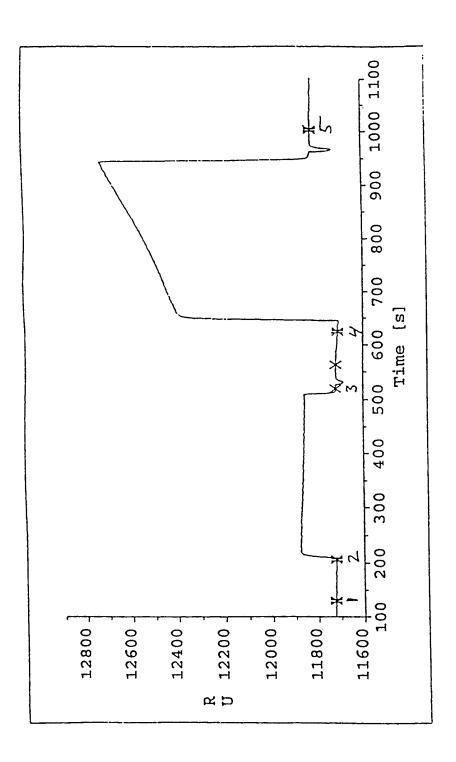




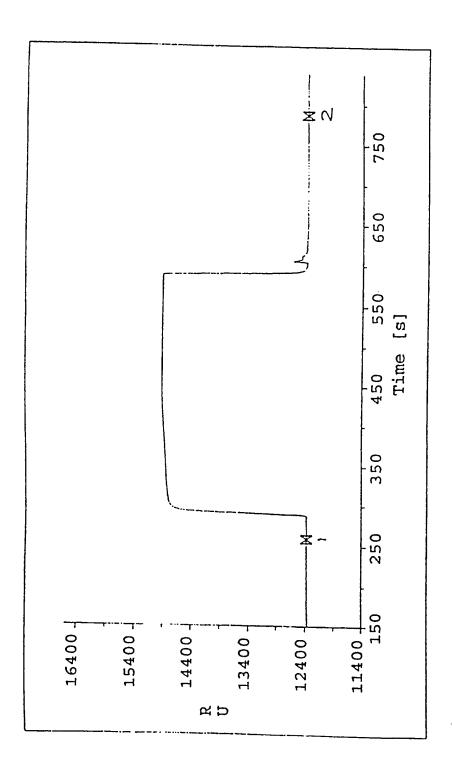


F16. 3

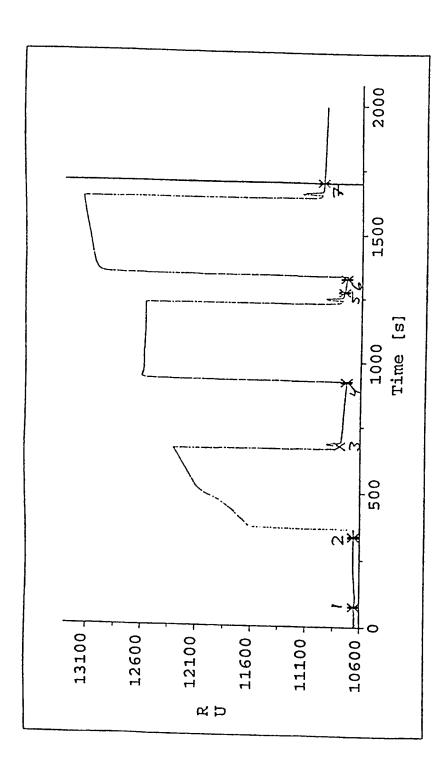




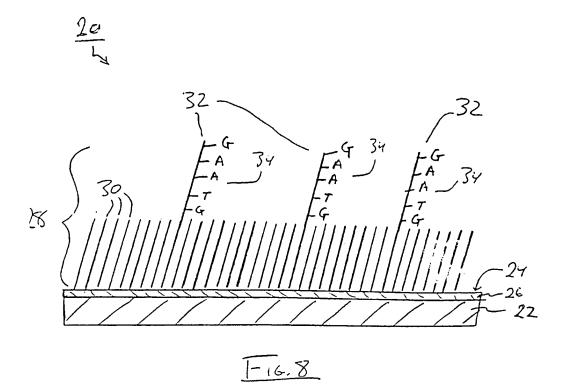
1-16.5

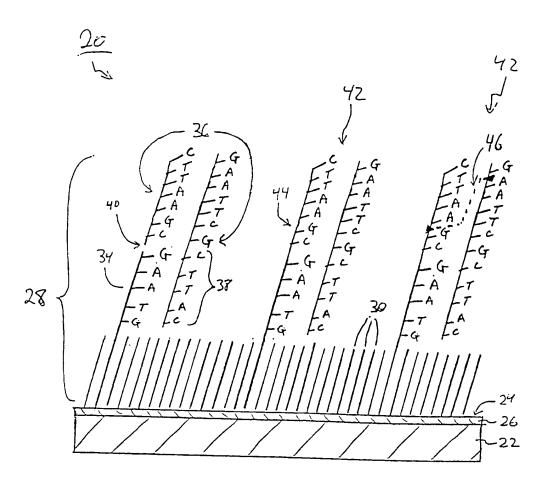


-16.6

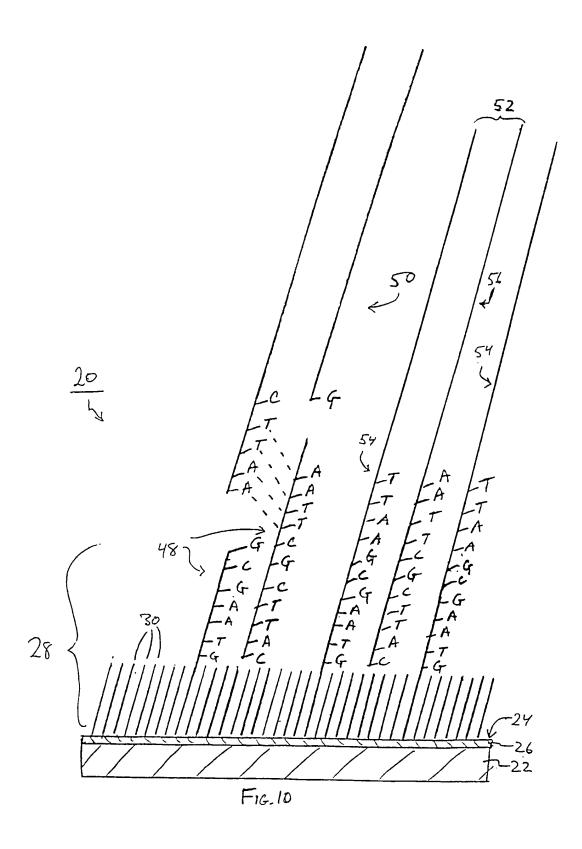


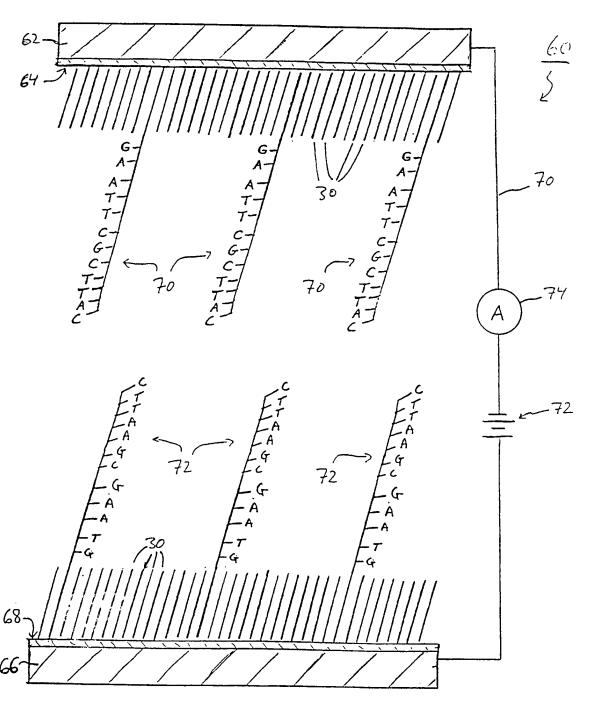
F16.7



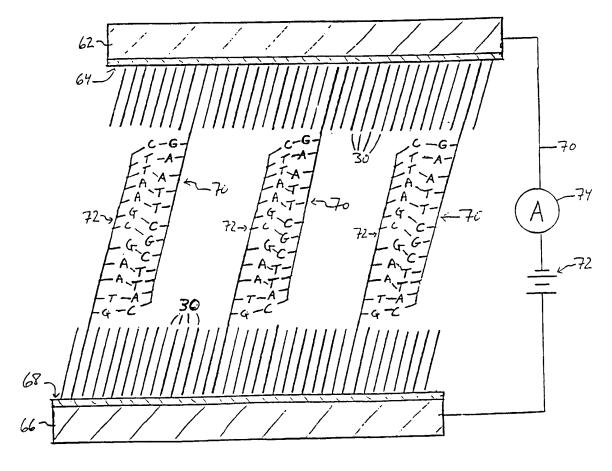


F16.9

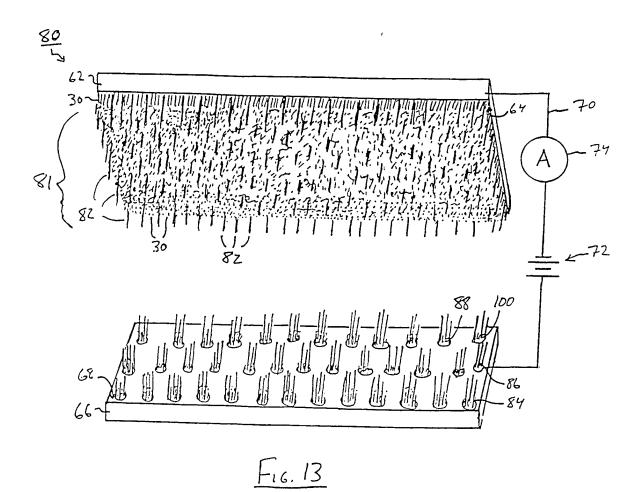


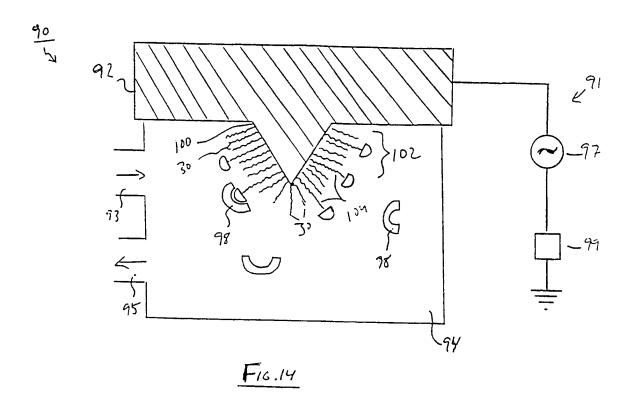


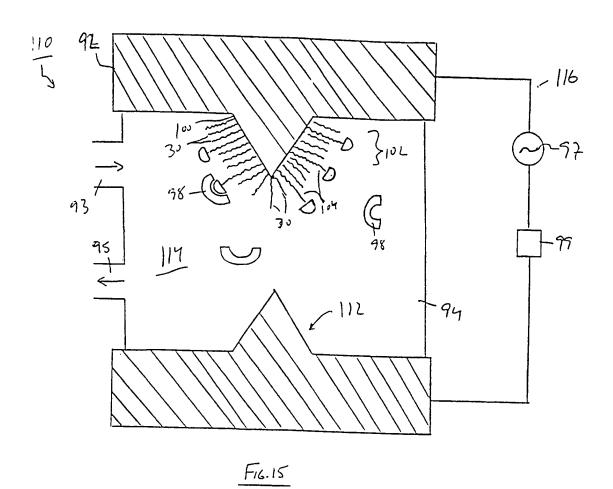
F16.11

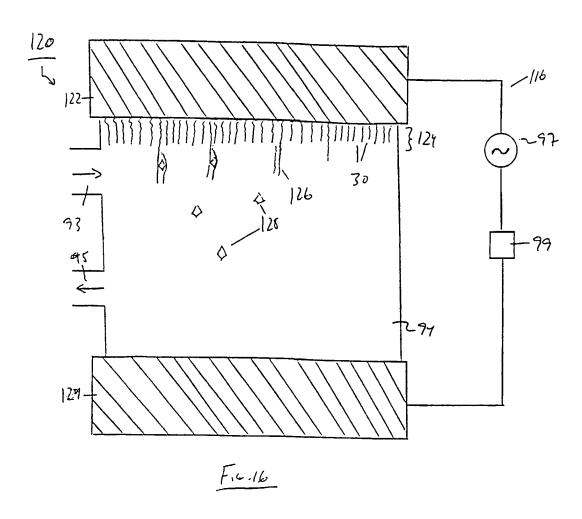


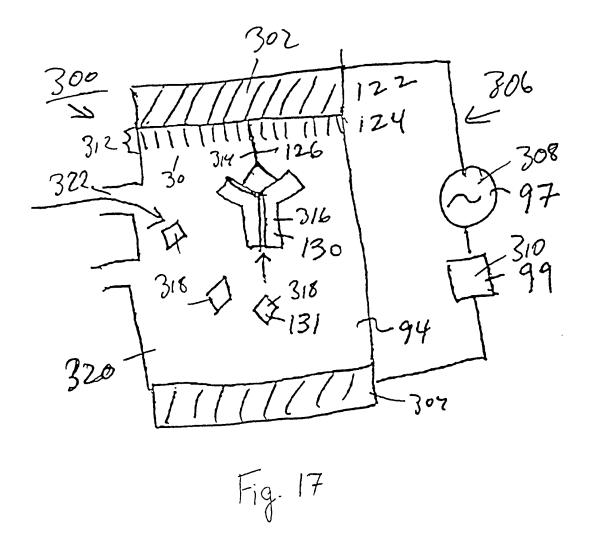
F16.12











PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :		(11) International Publication Number:	WO 98/31839
C12Q 1/68, G01N 33/53	A3	(43) International Publication Date:	23 July 1998 (23.07.98)

(21) International Application Number: PCT/US98/01144

(22) International Filing Date: 21 January 1998 (21.01.98)

(30) Priority Data:

 08/786,153
 21 January 1997 (21.01.97)
 US

 08/804,883
 24 February 1997 (24.02.97)
 US

 08/843,623
 10 April 1997 (10.04.97)
 US

(71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mount Auburn Street, Cambridge, MA 02138-5701 (US).

(72) Inventor: BAMDAD, Cynthia, C.; 621 Sierra Madre Boulevard, San Marino, CA 91108 (US).

(74) Agent: OYER, Timothy, J.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

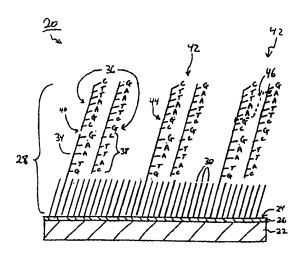
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

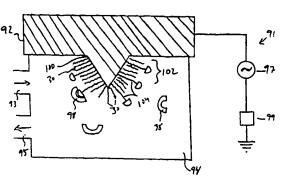
(88) Date of publication of the international search report: 17 September 1998 (17.09.98)

(54) Title: ELECTRONIC-PROPERTY PROBING OF BIOLOGICAL MOLECULES AT SURFACES

(57) Abstract

A technique for immobilizing biological molecules, in particular nucleic acid strands, is described. Biological molecules immobilized at surfaces can be used in electron-transfer detection techniques in which a binding partner of a biological molecule is brought into proximity of the surface-immobilized biological molecule, an electrical potential created between the two biologically-binding species, and electron transfer through the species determined. Another technique involves immobilizing a biological molecule such as a protein. DNA, etc., at a surface via a self-assembled monolayer, affecting the biological molecule via, for example, biological binding, inducing a change in conformation via a prion, etc., and detecting an electronic property change in the molecule via a change in impedance associated with an electronic circuit addressed by the biological molecule. This technique facilitates combinatorial array detection articles.





FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Annenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
	Burgaria Benin	(E	Ireland	MN	Mongolia	UA	Ukraine
BJ BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of Americ
	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CA	-	JP	Japan	NE	Niger	VN	Viet Nam
CF	Central African Republic	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CG	Congo	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CH	Switzerland	KP	Democratic People's	NZ	New Zealand		
CI	Côte d'Ivoire	K1	Republic of Korea	PL.	Poland		
CM	Cameroon	170	Republic of Korea	PT	Portugal		
CN	China	KR		RO	Romania		
CU	Cuba	KZ	Kazakstan Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LC	Liechtenstein	SD	Sudan		
DE	Germany	LI		SE SE	Sweden		
DK	Denmark	LK	Sri Lanka	SE SG			
EE	Estonia	LR	Liberia	36	Singapore		

INTERNATIONAL SEARCH REPORT

II 1ational Application No

			,
A. CLASS IPC 6	SIFICATION OF SUBJECT MATTER C1201/68 G01N33/53		
According	to International Patent Classification (IPC) or to both national class	sification and IPC	
	S SEARCHED		
Minimum di IPC 6	documentation searched (classification system followed by classific C 12Q	cation symbols)	
	ation searched other than minimum documentation to the extent th		
Electronic	data base consulted during the international search (name of data	a base and, where practical.	search terms used)
	MENTS CONSIDERED TO BE RELEVANT		
Category ,	Citation of document, with indication, where appropriate, of the	relevant passages	Relevant to claim No.
X	WO 93 22678 A (BAYLOR COLLEGE M; HOUSTON ADVANCED RES CENTER (UMASSACH) 11 November 1993 see whole document, esp. claims 13, line 9 ff	15);	1-12, 19-39
X	CUNNINGHAM B. C. &WELLS J. A.: "Comparison of a structural and functional epitope" J. MOL. BIOL., vol. 234, - 1993 pages 554-563, XP002071221 see esp. results page 555, 2.c figure la		40
	ther documents are listed in the continuation of box C.	X Patent family m	embers are listed in annex.
"A" docume consid "E" earlier of filing d "L" docume which crtatior "O" docume other r "P" docume	ategories of ofted documents: ient defining the general state of the art which is not dered to be of particular relevance document but published, on or other the international date ent which may throw double on printy claim(s) or it is cited to establish the publication date of another on or other special reason (as specified) entering to an oral disclosure, use, exhibition or means lent published prior to the international filling date but than the priority date claimed.	or priority date and cited to understand invention "X" document of particul cannot be consider involve an inventive "Y" document of particul cannot be consider document is combin	shed after the international filing date not in conflict with the application but the principle or theory underlying the ar relevance; the claimed invention ed novel or cannot be considered to be step when the document is taken alone ar relevance; the claimed invention ed to involve an inventive step when the ned with one or more other such document being obvious to a person skilled of the same patent family
	actual completion of theinternational search		e international search report
	.3 July 1998	27/07/19	98
Name and n	mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authonzed officer	F
	Fax. (+31-70) 340-3010	1,	•

Form PCT/ISA/210 (second sheet) (July 1992)

INTER. JONAL SEARCH REPORT

i. (atlonal Application No PCT/US 98/01144

	PCT/US 98/01144
ation) DOCUMENTS CONSIDERED TO BE RELEVANT	
Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
WO 97 44651 A (AUSTRALIAN MENBRANE & BIOTECH :UNIV SYDNEY (AU); HARDING MARGARET) 27 November 1997 see whole doc. esp. page 7 ,line 16 - page 8 ,line 19 and claims	1-12,20, 36
WO 97 41425 A (PENCE INC ;UNIV MCGILL (CA)) 6 November 1997 see whole document, esp. claims and figures	1-12,20, 36
MURPHY C.J. ET AL.,: "Long-range photoinduced electron transfer through a DNA helix" SCIENCE, vol. 262, - 12 November 1993 pages 1025-1029, XP002071222 cited in the application see esp. page 1028, 3. column	1-39
SIGAL G. B. ET AL.,: "A self-assembled monolayer for the binding and study of histidine-tagged proteins by surface plasmon resonance" ANAL. CHEM., - 1996 pages 490-497, XP002071235 see the whole document	13-18
"Kinetic characterization of DNA hybridization using real-time BIA" PHARMACIA BIOSENSOR, APPLICATION NOTES, no. 306, - 1994 XP002071223 see the whole document	1-39
NILSSON P. ET AL.,: "DNA sequencing with BIA" BIA JOURNAL, vol. 2, no. 2, - 1995 page 25 XP002071224 see the whole document	1-39
US 5 620 850 A (BAMDAD CYNTHIA C ET AL) 15 April 1997 see the whole document	13-38
NGUYEN J. ET AL.,: "Prion protein peptides induce alpha-helix to beta-sheet conformational transitions" BIOCHEMISRY, vol. 34, - 1995 pages 4186-44192, XP002071225 see esp. page 4191, last para.	40-51
	BIOTECH ; UNIV SYDNEY (AU); HARDING MARGARET) 27 November 1997 see whole doc. esp. page 7 , line 16 - page 8 , line 19 and claims

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. iational Application No PCT/US 98/01144

Patent document cited in search report		Publication date		atent family member(s)	Publication date	
WO 9322678	A	11-11-1993	EP JP US	0638173 A 7508831 T 5653939 A	15-02-1995 28-09-1995 05-08-1997	
WO 9744651	Α	27-11-1997	AU	2757897 A	09-12-1997	
WO 9741425	Α	06-11-1997	AU AU WO	2563897 A 2563997 A 9741424 A	19-11-1997 19-11-1997 06-11-1997	
US 5620850	Α	15-04-1997	NONE		دانه خود هما هما خود من المدان ال	

Form PCT/ISA/210 (patent family annex) (July 1992)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:

C12Q 1/68, G01N 27/327, 33/50

(11) International Publication Number:

WO 99/51778

(43) International Publication Date:

NL, PT, SE).

cisco, CA 94111 (US).

14 October 1999 (14.10.99)

(21) International Application Number:

PCT/US99/07650

A1

US

(22) International Filing Date:

7 April 1999 (07.04.99)

(30) Priority Data:

09/056,995

8 April 1998 (08.04.98)

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,

(74) Agent: THOMSON, William, E., Jr.; McCutchen, Doyle,

Brown & Enersen, Three Embarcadero Center, San Fran-

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application

09/056,995 (CIP)

Filed on

8 April 1998 (08.04.98)

(71) Applicant (for all designated States except US): CALIFORNIA INSTITUTE OF TECHNOLOGY [US/US]; Office of Technology Transfer, 1200 E. California Boulevard, MC 210-85. Pasadena, CA 91125 (US).

(72) Inventors; and

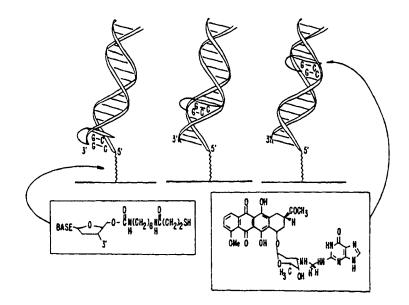
(75) Inventors/Applicants (for US only): BARTON, Jacqueline [US/US]; 1235 St. Albans Road San Marino, CA 91108 (US). HILL, Michael [US US]; 1516 East Del Mar, Pasadena, CA 91106 (US). KELLEY, Shana [US/US]; 1516 East Del Mar, Pasadena, CA 91106 (US).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ELECTROCHEMICAL SENSOR USING INTERCALATIVE, REDOX-ACTIVE MOIETIES



(57) Abstract

Compositions and methods for electrochemical detection and localization of genetic point mutations and other base-stacking perturbations within oligonucleotide duplexes adsorbed onto electrodes and their use in biosensing technologies are described. An intercalative, redox-active moiety (such as an intercalator or nucleic acid-binding protein) is adhered and/or cross-linked to immobilized DNA duplexes at different separations from an electrode and probed electrochemically in the presence or absence of a non-intercalative, redox-active moiety. Interruptions in DNA-mediated electron-transfer caused by base-stacking perturbations, such as mutations or binding of a protein to its recognition site, are reflected in a difference in electrical current, charge and/or potential.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Stovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and 1. Sago
BJ	Benin	Œ	Ireland	MN	Mongolia	UA	l'kraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Ug- rida
BY	Belarus	IS	Iceland	MW	Malawi	us	United States of America
CA	Canada	IТ	Italy	MX	Mexico	UZ	Uzhekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	КG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	ΚZ	Kazakstan	RO	Romania		
cz	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

PCT/US99/07650 WO 99/51778

1

ELECTROCHEMICAL SENSOR USING INTERCALATIVE, **REDOX-ACTIVE MOIETIES**

RELATED APPLICATION 1.

This application is a continuation-in part of Serial No. 60/043,146, filed April 9, 1997. 5

2. **GOVERNMENT RIGHTS**

The U.S. Government has certain rights in this invention pursuant to Grant No. GM 49216 awarded by the National Institute of Health.

FIELD OF THE INVENTION 3.

The present invention relates to the detection and localization of base-pair 10 mismatches and other perturbations in base-stacking within an oligonucleotide duplex.

4. DESCRIPTION OF RELATED ART

It is now well known that mutations in DNA can lead to severe consequences in metabolic functions (e.g., regulation of gene expression, modulation of protein production) which ultimately are expressed in a variety of diseases. For example, a significant number of human cancers are characterized by a single base mutation in one of the three ras genes (Bos, 1989). In order to unravel the genetic components of such diseases, it is of utmost importance to develop DNA sensors that are capable of detecting single-base mismatches rapidly and efficiently and to establish routine screening of disease-related genetic mutations based on such sensors (Skogerboe, 1993; Southern, 1996; Chee, 1996; Eng, 1997).

Various methods that have been developed for the detection of differences between DNA sequences rely on hybridization events to differentiate native versus mutated sequences and are limited by the small differences in base-pairing energies caused by point mutations within extended polynucleotides (Millan, 1993; Hashimoto, 1994; Xu, 1995; Wang, 1996; Lockhart, 1996; Alivisatos, 1996; Korriyoussoufi, 1997; Elghanian, 1997; Lin, 1997; Herne, 1997). Typically, a nucleic acid hybridization assay to determine the presence of a particular nucleotide sequence (i.e. the "target sequence") in either RNA or DNA comprises a multitude of steps. 30 First, an oligonucleotide probe having a nucleotide sequence complementary to at

15

20

10

15

20

25

30

least a portion of the target sequence is labeled with a readily detectable atom or group. When the labeled probe is exposed to a test sample suspected of containing the target nucleotide sequence, under hybridizing conditions, the target will hybridize with the probe. The presence of the target sequence in the sample can be determined qualitatively or quantitatively in a variety of ways, usually by separating the hybridized and non-hybridized probe, and then determining the amount of labeled probe which is hybridized, either by determining the presence of label in probe hybrids or by determining the quantity of label in the non-hybridized probes. Suitable labels may provide signals detectable by luminescence, radioactivity, colorimetry, xray diffraction or absorption, magnetism or enzymatic activity, and may include, for example, fluorophores, chromophores, radioactive isotopes, enzymes, and ligands having specific binding partners. However, the specific labeling method chosen depends on a multitude of factors, such as ease of attachment of the label, its sensitivity and stability over time, rapid and easy detection and quantification, as well as cost and safety issues. Thus, despite the abundance of labeling techniques, the usefulness, versatility and diagnostic value of a particular system for detecting a material of interest is often limited.

Some of the currently used methods of mismatch detection include single-strand conformation polymorphism (SSCP) (Thigpen, 1992; Orita, 1989), denaturing gradient gel electrophoresis (DGGE) (Finke, 1996; Wartell, 1990; Sheffield, 1989). RNase protection assays (Peltonen and Pulkkinen, 1986; Osborne, 1991), allelespecific oligonucleotides (Wu, 1989), allele-specific PCR (Finke, 1996), and the use of proteins which recognize nucleotide mismatches, such as the E. coli mutS protein (Modrich, 1991).

In the first three methods, the appearance of a new electrophoretic band is observed by polyacrylamide gel electrophoresis. SSCP detects the differences in speed of migration of single-stranded DNA sequences in polacrylamide gel electrophoresis under different conditions such as changes in pH, temperature, etc. A variation in the nucleotide base sequence of single-stranded DNA segments (due to mutation or polymorphism) may lead to a difference in spatial arrangement and thus in mobility. DGGE exploits differences in the stability of DNA segments in the

presence or absence of a mutation. Introduction of a mutation into double-stranded sequences creates a mismatch at the mutated site that destabilizes the DNA duplex. Using a gel with an increasing gradient of formamide (denaturation gradient gel), the mutant and wild-type DNA can be differentiated by their altered migration distances. The basis for the RNase protection assay is that the RNase A enzyme cleaves mRNA that is not fully hybridized with its complementary strand, whereas a completely hybridized duplex is protected from RNase A digestion. The presence of a mismatch results in incomplete hybridization and thus cleavage by RNase A at the mutation site. Formation of these smaller fragments upon cleavage can be detected by polvacrylamide gel electrophoresis. Techniques based on mismatch detection are generally being used to detect point mutations in a gene or its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of tumor samples. In addition to the RNase A protection assay, there are other DNA probes that can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Smooker and Cotton, 1993; Cotton, 1988; Shenk, 1975. Other enzymatic methods include for example the use of DNA ligase which covalently joins two adjacent oligonucleotides which are hybridized on a complementary target nucleic acid, see, for example Landegren (1988). The mismatch must occur at the site of ligation.

Alternatively, mismatches can also be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes (Cariello, 1988). With either riboprobes or DNA probes, the cellular mRNA or DNA which may contain a mutation can be amplified using polymerase chain reaction (PCR) prior to hybridization. Changes in DNA of the gene itself can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

DNA sequences of the specified gene which have been amplified by use of PCR may also be screened using allele-specific oligonucleotide probes. These probes are nucleic acid oligomers, each of which is complementary to a corresponding segment of the investigated gene and may or may not contain a known mutation. The assay is performed by detecting the presence or absence of a hybridization signal for

5

10

15

20

25

10

15

20

25

30

the specific sequence. In the case of allele-specific PCR, the PCR technique uses unique primers which selectively hybridize at their 3'-ends to a particular mutated sequence. If the particular mutation is not present, no amplification product is observed.

In addition, restriction fragment length polymorphism (RFLP) probes for the gene or surrounding marker genes can be used to score alteration of an allele or an insertion in a polymorphic fragment. However, since the recognition site of restriction endonucleases ranges in general between 4 to 10 base pairs, only a small portion of the genome is monitored by any one enzyme.

Another means for identifying base substitution is direct sequencing of a nucleic acid fragment. The traditional methods are based on preparing a mixture of randomly-terminated, differentially labeled DNA fragments by degradation at specific nucleotides, or by dideoxy chain termination of replicating strands (Maxam & Gilbert, 1980; Sanger, 1977). Resulting DNA fragments in the range of 1 to 500 bas specific are then separated on a gel to produce a ladder of bands wherein the adjacent samples differ in length by one nucleotide. The other method for sequencing nucleic acids is sequencing by hybridization (SBH, Drmanac, 1993). Using mismatch discriminative hybridization of short n-nucleotide oligomers (n-mers), lists of constitutent n-mers may be determined for target DNA. The DNA sequence for the target DNA may be assembled by uniquely overlapping scored oligonucleotides. Yet another approach relies on hybridization to high-density arrays of oligonucleotides to determine genetic variation. Using a two-color labeling scheme simultaneous comparison of a polymorphic target to a reference DNA or RNA can be achieved (Lipshutz, 1995; Chee, 1996; Hacia, 1996).

Each of these known prior art methods for detecting base pair mismatches has limitations that affect adequate sensitivity, specificity and ease of automation of the assay. In particular, these methods are unable to detect mismatches independent of sequence composition and require carefully controlled conditions, and most methods detect multiple mismatches only. Additional shortcomings that limit these methods include high background signal, poor enzyme specificity, and/or contamination.

10

15

20

25

30

Over the last decade, attention has also focused on DNA as a medium of charge transfer in photoinduced electron transfer reactions and its role in mutagenesis and carcinogenesis. For example, studies were performed using various octahedral metal complexes (which bind tightly to DNA by intercalation) as donors and acceptors for photoinduced electron transfer. Dppz complexes of ruthenium, osmium, cobalt, nickel, and rhenium showed tight intercalative binding and unique photophysical and electrochemical properties. No photoluminesence was observed upon irradiation of the metal complexes in aqueous solution in absence of DNA (as a result of quenching by proton transfer from the solvent), whereas in the presence of DNA excitation of the complex afforded significant, long-wavelength emission (because now the intercalated complex was protected from quenching). Studies using rhodium intercalators containing phenanthrenequinone-diimine (phi) ligands displayed tight DNA binding by preferential intercalation, some with affinities and specifities approaching DNA-binding proteins.

Photoinduced electron transfer using DNA as a molecular bridge has been established in various systems. Using metal complexes intercalated into the base stack of DNA as donor and acceptor it has been proposed that the DNA π -stack could promote electron transfer at long range. Additionally, the products of redox-triggered reactions of DNA bases have been detected at sites remote from intercalating oxidants (Hall, 1996; Dandliker, 1997; Hall, 1997; Arkin, 1997). For example, it has been shown that a metallointercalator can promote oxidative DNA damage through longrange hole migration from a remote site. Oligomeric DNA duplexes were prepared with a rhodium intercalator covalently attached to one end and separated spatially from 5'-GG-3' doublet sites of oxidation. Rhodium-induced photooxidation occurred specifically at the 5'-G in the 5'-GG-3' doublets and was observed up to 37 Å away from the site of rhodium intercalation. In addition it was found that rhodium intercalators excited with 400 nm light, initiated the repair of a thymine dimer incorporated site-specifically in the center of a synthetic 16-mer oligonucleotide duplex. The repair mechanism was thought to proceed via oxidation of the dimer by the intraligand excited state of the rhodium complex, in which an electron deficiency (hole) is localized on the intercalated phi ligand. Like electron transfer between

6

metallointercalators, the efficiencies of long-range oxidative processes were found to be remarkably sensitive to the coupling of the reactants into the base stack (Holmlin, 1997) and depended upon the integrity of the base stack itself (Kelley, 1997c, 1997d; Hall, 1997; Arkin, 1997) as well as on the oxidation potential. Perturbations caused by mismatches or bulges greatly diminished the yields of DNA-mediated charge transport.

Other studies have reported electron transfer through DNA using nonintercalating ruthenium complexes coordinated directly to amino-modified sugars at the terminal position of oligonucleotides (Meade, 1995). In this system it was suggested that electron transfer is protein-like. In proteins, where the energetic differences in coupling depend upon σ -bonded interactions, small energetic differences between systems do not cause large differences in electronic coupling. In the DNA double helix however, π -stacking can contribute to electronic coupling such that small energetic differences could lead to large differences in coupling efficiency. Most recently, Lewis and coworkers measured rates of photo-oxidation of a guanine base in a DNA hairpin by an associated stilbene bound at the top of the hairpin (Lewis, 1997). By systematically varying the position of the guanine base within the hairpin and measuring the rate of electron transfer, a value for β , the electronic coupling parameter, could be made. Here, β was found to be intermediate between that seen in proteins, with σ bonded arrays, and that found for a highly coupled π -bonded array.

Electrochemical studies of small molecule/DNA complexes have focused primarily on solution-phase phenomena, in which DNA-induced changes in redox potentials and/or diffusion constants of organic and inorganic species have been analyzed to yield association constants (Carter, 1989, 1990; Rodriguez, 1990; Welch, 1995; Kelly, 1986; Molinier-Jumel, 1978; Berg, 1981; Plambeck, 1984). In addition, rates of guanine oxidation catalyzed by electrochemically oxidized transition-metal complexes have been used to evaluate the solvent accessibility of bases for the detection of mismatches in solution (Johnston, 1995). Electrochemical signals triggered by the association of small molecules with DNA have also been applied in the design of other novel biosensors. Toward this end, oligonucleotides have been

5

10

15

20

25

7

immobilized on electrode surfaces by a variety of linkages for use in hybridization assays. These include thiols on gold (Hashimoto, 1994a, 1994b; Okahata, 1992), carbodiimide coupling of guanine residues on glassy carbon (Millan, 1993), and alkane bisphosphonate films on Al³⁺-treated gold (Xu, 1994, 1995). Both direct changes in mass (measured at a quartz crystal microbalance) (Okahata, 1992) and changes in current (Hashimoto, 1994a, 1994b; Millan, 1993) or electrogenerated chemiluminesence (Xu, 1994, 1995) due to duplex-binding molecules have been used as reporters for double stranded DNA. Gold surfaces modified with thiolated polynucleotides have also been used for the detection of metal ions and DNA-binding drugs (Maeda, 1992, 1994).

Other known electrochemical sensors used in an increasing number of clinical, environmental, agricultural and biotechnological applications include enzyme based biosensors. Amperometric enzyme electrodes typically require some form of electrical communication between the electrode and the active site of the redox enzyme that is reduced or oxidized by the substrate. In one type of enzyme electrode, a non-natural redox couple mediates electron transfer from the substrate-reduced enzyme to the electrode. In this scheme, the enzyme is reduced by its natural substrate at a given rate; the reduced enzyme is in turn, rapidly oxidized by a non-natural oxidizing component of a redox couple that diffuses into the enzyme, is reduced, diffuses out and eventually diffuses to an electrode where it is oxidized.

Electrons from a substrate-reduced enzyme will be transferred either to the enzyme's natural re-oxidizer or, *via* the redox-centers of the polymer to the electrode. Only the latter process contributes to the current. Thus, it is desirable to make the latter process fast relative to the first. This can be accomplished by (a) increasing the concentration of the redox centers, or (b) assuring that these centers are fast, i.e. that they are rapidly oxidized and reduced.

Most natural enzymes are not directly oxidized at electrodes without being destroyed, even if the latter are maintained at strongly oxidizing potentials. Also they are not reduced at strongly reducing potentials without being decomposed. It has, however, been shown that enzymes can be chemically modified by binding to their proteins redox couples, whereupon, if in the reduced state, they transfer electrons to

5

10

15

20

25

10

15

20

25

30

methods.

an electrode. It has also been shown that when redox polycations in solution electrostatically complex polyanionic enzymes, electrons will flow in these complexes from the substrate to the enzyme, and from the enzyme through the redox polymer, to an electrode. In addition, systems have been developed where a redox-active polymer, such as poly(vinyl-pyridine), has been introduced which electrically connects the enzyme to the electrode. In this case, the polycationic redox polymer forms electrostatic complexes with the polyanionic glucose oxidase in a manner mimicking the natural attraction of some redox proteins for enzymes, e.g., cytochrome c for cytochrome c oxidase.

The present invention provides a new approach for the detection of mismatches based on charge transduction through DNA. This electrochemical method is based on DNA-mediated electron transfer using intercalative, redox-active species and detects differences in electrical current or charge generated with fully base-paired duplexes *versus* duplexes containing a base-stacking perturbation, such as a mismatch. Carried out at an addressable multielectrode array, this method allows the processing of multiple sequences in the course of a single measurement, thus significantly improving the efficiency of screening for multiple genetic defects. Most importantly, the assay reports directly on the structural difference in base pair stacking within the hybridized duplex, rather than on a thermodynamic difference based on the condition-dependent hybridization event itself. Consequently, mismatch detection becomes independent of the sequence composition and sensors based on this approach offer fundamental advantages in both scope and sensitivity over any other existing

5. SUMMARY OF THE INVENTION

The present invention provides a highly sensitive and accurate method for the detection of genetic point mutations in nucleic acid sequences and its application as a biosensor. In particular, the invention relates to electrodes that are prepared by modifying their surfaces with oligonucleotide duplexes combined with an intercalative, redox-active species and their use as sensors based on an electrochemical process in which electrons are transferred between the electrode and the redox-active species.

9

One aspect of the invention relates to methods for determining the presence of point mutations sequentially in a series of oligonucleotide duplexes using an intercalative, redox-active moiety. A preferred method comprises the steps of: (a) contacting at least one strand of a first nucleic acid molecule with a strand of a second nucleic acid molecule under hybridizing conditions, wherein one of the nucleic acid molecules is derivatized with a functionalized linker, (b) depositing this duplex onto an electrode or an addressable multielectrode array, (c) contacting the adsorbed duplex which potentially contains a base-pair mismatch with an intercalative, redox-active moiety under conditions suitable to allow complex formation, (d) measuring the amount of electrical current or charge generated as an indication of the presence of a base-pair mismatch within the adsorbed duplex. (e) treating the complex under denaturing conditions in order to separate the complex, yielding a monolayer of single-stranded oligonucleotides, and (f) rehybridizing the single-stranded oligonucleotides with another target sequence. Steps (c) through (f) can then be repeated for a sequential analysis of various oligonucleotide probes. Attenuated signals, as compared to the observed signals for fully base-paired, i.e. wild-type, sequences, will correspond to mutated sequences.

In some instances, it may be desirable to crosslink the intercalative, redoxactive species to the duplex and perform the assay comprised of steps (a) through (d) only.

Another preferred method relates to the detection of point mutations utilizing electrocatalytic principles. More specifically, this method utilizes an electrode-bound double-stranded DNA monolayer which is immersed in a solution comprising an intercalative, redox-active species, which binds to the monolayer surface, and a non-intercalative redox-active species which remains in solution. This method comprises the steps of: (a) contacting at least one strand of a first nucleic acid molecule with a strand of a second nucleic acid molecule under hybridizing conditions, wherein one of the nucleic acid molecules is derivatized with a functionalized linker, (b) depositing this duplex which potentially contains a base-pair mismatch onto an electrode or an addressable multielectrode array. (c) immersing this complex in an aqueous solution comprising an intercalative, redox-active moiety and a non-intercalative, redox-active

5

10

15

20

25

10

15

20

25

30

moiety under conditions suitable to allow complex formation, (d) measuring the amount of electrical current or charge generated as an indication of the presence of a base-pair mismatch within the adsorbed duplex, (e) treating the complex under denaturing conditions in order to separate the complex, yielding a monolayer of single-stranded oligonucleotides, and (f) rehybridizing the single-stranded oligonucleotides with another target sequence. Steps (c) through (f) can then be repeated for a sequential analysis of various oligonucleotide probes. Utilizing this method, pronounced currents and thus increased signals will be observed due to the electrocatalytic reduction of the non-intercalative, redox-active moiety by the surface-bound, redox-active moiety.

Yet another aspect of the invention relates to a method of detecting the presence or absence of a protein and comprises the steps of: (a) contacting at least one strand of a first nucleic acid molecule with a strand of a second nucleic acid molecule under hybridizing conditions, wherein one of the nucleic acid molecules is der varized with a functionalized linker and wherein the formed duplex is designed such to contain the recognition site of a nucleic acid-binding protein of choice, (b) depositing this duplex onto an electrode or an addressable multielectrode array, (c) contacting the adsorbed duplex with an intercalative, redox-active moiety under conditions suitable to allow complex formation, (d) potentially crosslinking the intercalative, redox-active moiety to the duplex, (e) immersing the complex in a first sample solution to be analyzed for the presence of the nucleic acid-binding protein, (f) measuring the amount of electrical current or charge generated as an indication of the presence or absence of the nucleic acid-binding protein in the sample solution, (g) treating the complex under appropriate conditions to remove the nucleic acid-binding protein, and (h) immersing it in a second sample solution to be analyzed for the presence of the nucleic acid-binding protein in order to separate the complex. Steps (e) through (h) can then be repeated for a sequential analysis of various sample solutions. Attenuated signals, as compared to signals measured for a reference solution without the nucleic acid-binding protein, indicate the presence of the nucleic acid-binding protein which is binding to its recognition site, thus causing a perturbation in base-stacking.

11

The invention also relates to the nature of the redox-active moieties. The requirements of a suitable intercalative, redox-active moiety include the position of its redox potential with respect to the window within which the oligonucleotide-surface linkage is stable, as well as the synthetic feasibility of covalent attachment to the oligonucleotide. In addition, chemical and physical characteristics of the redox-active intercalator may promote its intercalation in a site-specific or a non-specific manner. In a preferred embodiment, the redox-active species is in itself an intercalator or a larger entity, such as a nucleic acid-binding protein, that contains an intercalative moiety.

The nature of the non-intercalative, redox-active species for the electrocatalysis based assays depends primarily on the redox potential of the intercalative, redox-active species utilized in that assay.

Yet another aspect of the invention relates to the composition and length of the oligonucleotide probe and methods of generating them. In a preferred embodiment, the probe is comprised of two nucleic acid strands of equal length. In another preferred embodiment the two nucleic acid strands are of uneven length, generating a single-stranded overhang of desired sequence composition (i.e. a "sticky end"). The length of the oligonucleotide probes range preferably from 12 to 25 nucleotides, while the single-stranded overhangs are approximately 5 to 10 nucleotides in length. These single-stranded overhangs can be used to promote site-specific adsorption of other oligonucleotides with the complementary overhang or of enzymes with the matching recognition site.

The invention further relates to methods of creating a spatially addressable array of adsorbed duplexes. A preferred method comprises the steps of (a) generating duplexes of variable sequence composition that are derivatized with a functionalized linker, (b) depositing these duplexes on different sites on the multielectrode array, (c) treating the complex under denaturing conditions to yield a monolayer of single-stranded oligonucleotides, and (d) hybridizing these single-stranded oligonucleotides with a complementary target sequence. Another preferred method comprises the steps of (a) depositing 5 to 10 base-pair long oligonucleotide duplexes that are derivatized on one end with a functionalized linker and contain single-stranded overhangs

5

10

15

20

25

(approximately 5 to 10 nucleotides long) of known sequence composition at the opposite end onto a multielectrode array, and (b) contacting these electrode-bound duplexes under hybridizing conditions with single-stranded or double-stranded oligonucleotides that contain the complementary overhang.

Another aspect of the invention is directed towards the nature of the electrode, methods of depositing an oligonucleotide duplex (with or without a redox-active moiety adsorbed to it) onto an electrode, and the nature of the linkage connecting the oligonucleotide duplex to the electrode. In a preferred embodiment, the electrode is gold and the oligonucleotide is attached to the electrode by a sulfur linkage. In another preferred embodiment the electrode is carbon and the linkage is a more stable amide bond. In either case, the linker connecting the oligonucleotide to the electrode is preferably comprised of 5 to 20 σ bonds.

Yet another aspect of the invention relates to various methods of detection of the electrical current or charge generated by the electrode-bound duplexes combined with an intercalative, redox-active species. In a preferred embodiment, the electrical current or charge is detected using electronic methods, for example voltammetry or amperommetry, or optical methods, for example fluorescence or phosphoresence. In another preferred embodiment, the potential at which the electrical current is generated is detected by potentionmetry.

20 6. BRIEF DESCRIPTION OF DRAWINGS

Table 1 describes the electrochemical detection of single-base mismatches based on cyclic voltammograms measured for 1.0 μM daunomycin noncovalently bound to duplex-modified electrodes.

Figure 1 is a schematic diagram depicting DNA duplexes used for study of distance-dependent reduction of daunomycin. The right insert illustrates the daunomycin-guanine crosslink. The left insert shows the thiol-terminated tether which connects the duplex to the electrode surface and provides $16\ \sigma$ -bonds between the electrode and the base stack.

Figure 2 illustrates cyclic voltammograms of gold electrodes modified with daunomycin-crosslinked thiol-terminated duplexes (A) SH-5 ATGGATCTCATCTAC

5

10

15

+ complement and (B) SH-⁵ ATCCTACTCATGGAC + complement, where the bold Gs represent the daunomycin crosslinking site.

Figure 3 illustrates cyclic voltammograms of gold electrodes modified with daunomycin-crosslinked thiol-terminated duplexes containing TA and CA basepairs.

The oligonucleotide SH-⁵ ATTATATATAATTGCT was hybridized with the corresponding complements containing either a T or a C opposite from the underlined A.

Figure 4 describes the charges (Q_c) measured for daunomycin at DNA-modified electrodes containing different single-base mismatches. To obtain the seven different mismatched duplexes the thiol-modified sequence, SH-

SAGTACAGTCATCGCG, was hybridized with the following seven different complements (the mismatch is indicated in bold, and the specific basepair and the melting temperature of the duplex is given in parentheses): 5 CGCGATGACTGTACT (TA, $T_m = 68$ °C), 5 CGCGACGACTGTACT (CA, $T_m = 56$ °C),

5 CGCGATGTCTGTACT (TT, T_m = 57 °C), 5 CGCGATGACTGTACT (CC, T_m = 56 °C), 5 CGCGATGGCTGTACT (GT, T_m = 62 °C), 5 CGCGATGAATGTACT (GA, T_m = 60 °C), 5 CGCGATGCCTGTACT (CT, T_m = 58 °C).

Figure 5 describes the charge obtained for DNA-modified electrodes in the presence of 1.0 μ M daunomycin. the identified duplexes of varying percentages of GC content were either fully base-paired or contained a single CA mismatch. Mismatch detection measuring the electrical current or charge generated was independent of the sequence composition.

Figure 6 describes the charges (Q_c) measured during the *in situ* detection of a CA mismatch. Electrodes were derivatized with the sequence SH-

25 SAGTACAGTCATCGCG, where either a C or a T was incorporated into the complement across from the underlined A. Using cyclic voltammetry, the electrochemical response of daunomycin non-covalently bound to duplex-modified electrodes was measured first for the intact TA or CA duplexes (TA vs. CA), secondly (after denaturation of the duplex) for the single stranded oligonucleotide (ss), thirdly
30 (after rehybridization with the opposite complement) again for the duplex (CA vs.

10

TA), and lastly (after repeating the denaturation step) again for the single-stranded oligonuclectide (ss).

Figure 7 represents a schematic illustration of electrocatalytic reduction of ferricyanide. Methylene blue (MB⁺) is reduced electrochemically through the DNA base stack to form leucomethylene blue (LB⁺). Ferricyanide is then reduced by LB⁺, causing the regeneration of MB⁺ and the observation of catalytic currents.

Figure 8 illustrates cyclic voltammograms of gold electrodes modified with thiol-terminated duplexes containing TA and CA basepairs immersed in a solution containing 1.0 µM methylene blue and 1.0 mM ferricyanide. The oligonucleotide SH- STACAGTCATCGCG was hybridized with the corresponding complements containing either a T or a C opposite from the underlined A.

Figure 9 shows chronocoloumetry at -350 mV of 2.0 mM Fe(CN)₆³⁻ plus 20.5 μM methylene blue (pH 7) at a gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG-3' hybridized to a fully base-paired complement (upper trace) and a complement that features an A opposite the bold C (lower trace). The discrimination between base-paired and mismatched sequences increases with increased sampling time.

7. DETAILED DESCRIPTION OF THE INVENTION

20 polymerase chain reaction (PCR), ligation amplification (or ligase chain reaction, LCR) and amplification methods based on the use of Q-beta replicase. These methods are well known and widely practiced in the art. See, e.g., U.S. Pat. Nos. 4.683,195 and 4.683,202 and Innis et al., 1990 (for PCR); and Wu et al., 1989a (for LCR). Reagents and hardware for conducting PCR are commercially available. Primers useful to amplify sequences from a particular gene region are preferably complementary to, and hybridize specifically to sequences in the target region or in its flanking regions. Nucleic acid sequences generated by amplification may be sequenced directly. Alternatively the amplified sequence(s) may be cloned prior to sequence analysis. A method for the direct cloning and sequence analysis of enzymatically amplified genomic segments has been described by Scharf (1986).

10

15

The term "base-stacking perturbations" refers to any event that causes a perturbation in base-stacking such as, for example, a base-pair mismatch, a protein binding to its recognition site, or any other entities that form oligonucleotide adducts.

The term "denaturing" refers to the process by which strands of oligonucleotide duplexes are no longer base-paired by hydrogen bonding and are separated into single-stranded molecules. Methods of denaturation are well known to those skilled in the art and include thermal denaturation and alkaline denaturation.

The term "hybridized" refers to two nucleic acid strands associated with each other which may or may not be fully base-paired.

The term "intercalative moieties" refers to planar aromatic or heteroaromatic moieties that are capable of partial insertion and stacking between adjacent base pairs of double-stranded oligonucleotides. These moieties may be small molecules or part of a larger entity, such as a protein. Within the context of this invention the intercalative moiety is able to generate a response or mediate a catalytic event.

The term "mismatches" refers to nucleic acid bases within hybridized duplexes which are not 100% complementary. A mismatch includes any incorrect pairing between the bases of two nucleotides located on complementary strands of DNA that are not the Watson-Crick base-pairs A:T or G:C. The lacl: of total homology may be due to deletions, insertions, inversions, substitutions or frameshift mutations.

The term "mutation" refers to a sequence rearrangement within DNA. The most common single base mutations involve substitution of one purine or pyrimidine for the other (e.g., A for G or C for T or vice versa), a type of mutation referred to as a "transition". Other less frequent mutations include "transversions" in which a purine is substituted for a pyrimidine, or *vice versa*, and "insertions" or "deletions", respectively, where the addition or loss of a small number (1, 2 or 3) of nucleotides arises in one strand of a DNA duplex at some stage of the replication process. Such mutations are also known as "frameshift" mutations in the case of insertion/deletion of one of two nucleotides, due to their effects on translation of the genetic code into proteins. Mutations involving larger sequence rearrangement also may occur and can be important in medical genetics, but their occurrences are relatively rare compared to the classes summarized above.

5

10

15

20

25

10

15

20

25

The term "nucleoside" refers to a nitrogenous heterocyclic base linked to a pentose sugar, either a ribose, deoxyribose, or derivatives or analogs thereof. The term "nucleotide" relates to a phosphoric acid ester of a nucleoside comprising a nitrogenous heterocyclic base, a pentose sugar, and one or more phosphate or other backbone forming groups; it is the monomeric unit of an oligonucleotide. Nucleotide units may include the common bases such as guanine (G), adenine (A), cytosine (C), thymine (T), or derivatives thereof. The pentose sugar may be deoxyribose, ribose, or groups that substitute therefore.

The terms "nucleotide analog", "modified base", "base analog", or "modified nucleoside" refer to moieties that function similarly to their naturally occurring counterparts but have been structurally modified.

The terms "oligonucleotide" or "nucleotide sequence" refers to a plurality of joined nucleotide units formed in a specific sequence from naturally occurring heterocyclic bases and pentofuranosyl equivalent groups joined through phosphorodiester or other backbone forming groups.

The terms "oligonucleotide analogs" or "modified oligonucleotides" refer to compositions that function similarly to natural oligonucleotides but have non-naturally occurring portions. Oligonucleotide analogs or modified oligonucleotides may have altered sugar moieties, altered bases, both altered sugars and bases or altered inter-sugar linkages, which are known for use in the art.

The terms "redox-active moiety" or "redox-active species" refers to a compound that can be oxidized and reduced, i.e. which contains one or more chemical functions that accept and transfer electrons.

The term "redox protein" refers to proteins that bind electrons reversibly. The simplest redox proteins, in which no prosthetic group is present, are those that use reversible formation of a disulfide bond between to cysteine residues, as in thioredoxin. Most redox proteins however use prosthetic groups, such as flavins or NAD. Many use the ability of iron or copper ions to exist in two different redox states.

The present invention provides a highly sensitive and accurate method based on an electrochemical assay using intercalative, redox-active species to determine the

17

presence and location of a single or multiple base-pair mismatches. Briefly, the system is comprised of (i) a reagent mixture comprising an electrode-bound oligonucleotide duplex to which an intercalative, redox-active moiety is associated and (ii) means for detecting and quantitating the generated electrical current or charge as an indication for the presence of a fully base-paired *versus* a mismatch containing duplex. The present invention is particularly useful in the diagnosis of genetic diseases that arise from point mutations. For example, many cancers can be traced to point mutations in kinases, growth factors, receptors binding proteins and/or nuclear proteins. Other diseases that arise from genetic disorders include cystic fibrosis, Bloom's syndrome, thalassemia and sickle cell disease. In addition, several specific genes associated with cancer, such as DCC, NF-1, RB, p53, erbA and the Wilm's tumor gene, as well as various oncogenes, such as abl, erbB, src, sis, ras, fos, myb and myc have already been identified and examined for specific mutations.

The present invention provides methods for detecting single or multiple point mutations, wherein the oligonucleotide duplex carrying the redox-active species is adsorbed and therefore continuously exposed to an electrode whose potential oscillates between a potential sufficient to effect the reduction of said chemical moiety and a potential sufficient to effect the oxidation of the chemical moiety. This method is preferred over other methods for many reasons. Most importantly, this method allows the detection of one or more mismatches present within an oligonucleotide duplex based on a difference in electrical current measured for the mismatch-containing *versus* the fully base-paired duplex. Thus the method is based on the differences in base-stacking of the mismatches and is independent of the sequence composition of the hybridized duplex, as opposed to existing methods that depend on thermodynamic differences in hybridization. Furthermore, this method is nonhazardous, inexpensive, and can be used in a wide variety of applications, along or in combination with other hybridization-dependent methods.

One particular aspect of the invention relates to the method for sequential detection of mismatches within a number of nucleic acid samples which comprises the following steps. At least one strand of a nucleic acid molecule is hybridized under suitable conditions with a first nucleic acid target sequence forming a duplex which

5

10

15

20

25

10

15

20

25

30

potentially contains a mismatch, and wherein one of the nucleic acids is derivatized with a functionalized linker. This duplex is then deposited onto an electrode or an addressable multielectrode array forming a monolayer. An intercalative, redox-active species (e.g., daunomycin) is noncovalently adsorbed (or crosslinked, if desired) onto this molecular lawn, and the electrical current or charge generated is measured as an indication of the presence of a base pair mismatch within the adsorbed oligonucleotide complex. Subsequent treatment of the duplexes containing the intercalative, redox-active species under denaturing conditions allows separation of the complex, yielding a single-stranded monolayer of oligonucleotides which can be rehybridized to a second oligonucleotide target sequence. The steps of duplex formation, adsorption of the intercalative, redox-active species, measurement of the electrical current or charge, and denaturation of the complex to regenerate the single-stranded oligonucleotides may be repeated as often as desired to detect in a sequential manner genetic point mutations in a variety of oligonucleotide probes.

The charges passed at each of the electrodes is measured and compared to the wild-type, i.e. fully base-paired, sequences. Electrodes with attenuated signals correspond to mutated sequences, while those which exhibit no change in electrical current or charge are unmutated. Furthermore, the intensity of the signal compared to the wild-type sequence not only reports the presence of the mismatch but also describes the location of the disruption within the analyzed duplex.

Another aspect of the invention relates to the method of detecting mutations utilizing electrocatalysis. Briefly, the modification of electrode surfaces with oligonucleotide duplexes provides a medium that is impenetrable by negatively charged species due to the repulsion by the high negative charge of oligonucleotides. However, electrons can be shuttled through the immobilized duplexes to redox-active intercalators localized on the solvent-exposed periphery of the monolayer, which in turn can catalytically reduce these negatively charged species. More specifically, this electrocatalytic method comprises the following steps. At least one strand of a nucleic acid molecule is hybridized under suitable conditions with a first nucleic acid target sequence forming a duplex which potentially contains a mismatch, and wherein one of the nucleic acids is derivatized with a functionalized linker. This duplex is then

deposited onto an electrode or a multielectrode array forming a monolayer. The assembly is immersed into an aqueous solution containing both an intercalative, redox-active species (e.g., methylene blue) and a non-intercalative, redox-active species (e.g., ferricyanide). The electrical currents or charges corresponding to the catalytic reduction of ferricyanide mediated by methylene blue are measured for each nucleic acid-modified electrode and compared to those obtained with wild-type, i.e. fully base-paired sequences. Subsequent treatment of the duplexes under denaturing conditions allows separation of the complex, yielding a single-stranded monolayer of oligonucleotides which can be rehybridized to a second oligonucleotide target sequence. The steps of duplex formation, measurement of the catalytically enhanced electrical current or charge, and denaturation of the complex to regenerate the singlestranded oligonucleotides may be repeated as often as desired to detect in a sequential manner genetic point mutations in a variety of oligonucleotide probes. This particular method based on electrocatalysis at oligonucleotide-modified surfaces is extremely useful for systems where attenuated signals resulting from the presence of mismatches are small. The addition of a non-intercalative electron acceptor amplifies the signal intensity, and allows more accurate measurements. This approach may be particularly useful to monitor assays based on redox-active proteins which bind to the oligonucleotide-modified surface, but are not easily oxidized or reduced because the redox-active center is not intercalating.

The present invention further relates to the nature of the redox-active species. These species have a reduced state in which they can accept electron(s) and an oxidized state in which they can donate electron(s). The intercalative, redox-active species that are adsorbed or covalently linked to the oligonucleotide duplex include, but are not limited to, intercalators and nucleic acid-binding proteins which contain a redox-active moiety.

An intercalator useful for the specified electrochemical assays is an agent or moiety capable of partial insertion between stacked base pairs in the nucleic acid double helix. Examples of well-known intercalators include, but are not limited to, phenanthridines (e.g., ethidium), phenothiazines (e.g., methylene blue), phenazines (e.g., phenazine methosulfate), acridines (e.g., quinacrine), anthraquinones (e.g.,

5

10

15

20

25

20

daunomycin), and metal complexes containing intercalating ligands (e.g., phi, chrysene, dppz). One example of a suitable metal complex is the octahedral metal complex, Ir(bpy)(phen)(phi)³⁺. Some of these intercalators may interact site-selectively with the oligonucleotide duplex. For example, the chrysene ligand is known to intercalate at the mispaired site of a duplex itself (Jackson, 1997), which can be exploited for selective localization of an intercalator. This can be in particular useful to construct a duplex monolayer which contains the intercalative, redox-active species exclusively at its periphery.

In the case of redox-active nucleic acid-binding proteins, differences in DNA-mediated electron transfer between the duplex-bound protein and the electrode allow for the detection of base-pair mismatches or other base-stacking perturbations. Examples of redox-active proteins include, but are not limited to, mut Y, endonuclease III, as well as any redox-active cofactor-containing DNA-binding protein. Such proteins convert to one of an oxidized and reduced form upon reacting with a selected substrate, whereafter the operation of the electrode regenerates the other of the oxidized and reduced forms.

The choice of a protein depends partially on its adsorption and binding properties to biological macromolecules, i.e. nucleic acids, and with non-biological macromolecules, whether in a homogeneous solution, or when immobilized on a surface. By changing the absorption or binding characteristics, selectivity, signal to noise ratio and signal stability in these assays can be improved. The charge of a protein affects its adsorption on surfaces, absorption in films, electrophoretic deposition on electrode surfaces, and interaction with macromolecules. It is, therefore, of importance in diagnostic and analytical systems utilizing proteins to tailor the charge of the protein so as to enhance its adsorption or its binding to the macromolecule of choice, i.e the nucleic acid. In other cases, i.e when the detection assay is used during several cycles, it is of equal importance to be able to facilitate desorption, removal, or stripping of the protein from the macromolecule. These assays require oligonucleotide duplexes that are designed such as to allow for site-specific binding of the protein of choice, which may require a single-stranded

5

10

15

20

25

21

overhang. Once the protein is adsorbed onto the nucleic acid, electrons are relayed *via* the oligonucleotide duplex to the electrode.

The nature of the non-intercalative, redox-active species used in a particular electrocatalytic assay depends primarily on the redox potential of the intercalating, redox-active species utilized in that same assay. Examples include, but are not limited to, any neutral or negatively charged probes, for example ferricyanide/ferrocyanide, ferrocene and derivatives thereof (e.g., dimethylaminomethyl-, monocarboxylic acid-, dicarboxylic acid-), hexacyanoruthenate, and hexacyanoosmate.

Yet another aspect of the invention relates to a method of detecting the presence or absence of a protein inducing base-stacking perturbations in DNA duplexes, this method comprising the following steps. At least one strand of a nucleic acid molecule is hybridized under suitable conditions with a second strand of nucleic acid molecule forming a duplex, wherein one of the nucleic acids is derivatized with a functionalized linker. This duplex is designed such to contain the recognition site of a protein of choice at a distinct site along that duplex. This duplex is then deposited onto an electrode or an addressable multielectrode array forming a monolayer and an intercalative, redox-active species is adsorbed onto this molecular lawn. In a preferred embodiment, the intercalative, redox-active species is site-specifically localized. In another preferred embodiment, the intercalative, redox-active species is crosslinked to the oligonucleotide duplex. This formed complex is then exposed to a sample solution that potentially contains the specific protein and the electrical current or charge generated is measured as an indication of the presence or absence of the protein. Subsequently, the protein is removed under appropriate conditions to regenerate the duplex containing the intercalative, redox-active moiety. The steps of duplex formation, adsorption or crosslinking of the intercalative, redox-active species, measurement of the electrical current or charge, and regeneration of the duplex containing the intercalative, redox-active moiety may be repeated as often as desired to detect in a sequential manner the presence of a specific protein in multiple sample solutions.

The charges passed at each of the electrodes are measured and compared to the charges measured in a reference solution without the protein. Electrodes with

10

15

20

25

10

15

20

25

30

attenuated signals indicate the presence of the protein in question which is binding to its recognition site, thus causing a perturbation in base-stacking. Examples of proteins that can be used for this assay include, but are not limited to, restriction enzymes, TATA-binding proteins, and base-flipping enzymes (e.g., DNA methylase).

The present invention also relates to the choice of nucleic acid probes. Any nucleic acid, DNA or RNA, can be subjected to this mismatch detection method, provided that the mismatch(es) to be detected lie within the region between the attachment site of the intercalative, redox-active moiety and the electrode in order to be able to measure a difference in electrical current. The nucleic acid probes to be compared may comprise natural or synthetic sequences encoding up to the entire genome of an organism. These probes can be obtained from any source, for example, from plasmids, cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, organelles and higher organisms such as plants and animals. The samples may be extracted from tissue material or cells, including blood cells, amniocytes, bone marrow cells, cells obtained from a biopsy specimen and the like, by a variety of techniques as described for example by Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Cold Spring Harbor, New York (1982), incorporated herein by reference.

Alternatively, the sequences of choice can also be prepared by well known synthetic procedures. For standard DNA and RNA synthesis methods, see for example "Synthesis and Applications of DNA and RNA" ed. S. A. Narang, Academic Press, 1987, M. J. Gait, "Oligonucleotide Synthesis", IRL Press, Wash. D.C. U.S.A., 1984, and "Oligonucleotides and Analogues" ed. F. Eckstein, IRL Press, Wash. D.C. U.S.A., 1991, as incorporated herein by reference. Briefly, oligonucleotides and oligonucleotide analogs may be synthesized, conveniently through solid state synthesis of known methodology. In a preferred embodiment, the monomeric units are added to a growing oligonucleotide chain which is covalently immobilized to a solid support. Typically, the first nucleotide is attached to the support through a cleavable linkage prior to the initiation of synthesis. Step-wise extension of the oligonucleotide chain is normally carried out in the 3' to 5' direction. When the synthesis is complete, the polymer is cleaved from the support by hydrolyzing the

23

linkage mentioned above and the nucleotide originally attached to the support becomes the 3' terminus of the resulting oligomer. Nucleic acid synthesizers such as the Applied Biosystems, Incorporated 380B are commercially available and their use is generally understood by persons of ordinary skill in the art as being effective in generating nearly any oligonucleotide or oligonucleotide analog of reasonable length which may be desired. Triester, phosphoramidite, or hydrogen phosphonate coupling chemistries are used with these synthesizers to provide the desired oligonucleotides or oligonucleotide analogs.

In addition, the invention also relates to nucleic acid probes that are constructed with a defined sequence comprised of nucleotide and non-natural nucleotide monomers to restrict the number of binding sites of the intercalative, redox-active agent to one single site. For example, in the case of the redox-active intercalator daunomycin mixed nucleotide/non-natural nucleotide oligomers were prepared containing A-T and/or I-C basepairs and one discrete guanine binding site to which daunomycin is crosslinked. The non-natural nucleotides are constructed in a step-wise fashion to produce a mixed nucleotide/non-natural nucleotide polymer employing one of the current DNA synthesis methods well known in the art, see for example "Synthesis and Applications of DNA and RNA" ed. S. A. Narang, Academic Press, 1987, M. J. Gait, "Oligonucleotide Synthesis", IRL Press, Wash. D.C. U.S.A., 1984, and "Oligonucleotides and Analogues" ed. F. Eckstein, IRL Press, Wash. D.C. U.S.A., 1991.

Methods and conditions used for contacting the oligonucleotide strands of two DNAs, two RNAs or one DNA and one RNA molecule under hybridizing conditions are widely known in the art. Suitable hybridization conditions may be routinely determined by optimization procedures well known to those skilled in the art to establish protocols for use in a laboratory. See e.g., Ausubel et al., Current Protocols in Molecular Biology, Vol. 1-2, John Wiley & Sons (1989); Sambrook et al., Molecular Cloning A Laboratory Manual, 2nd Ed., Vols. 1-3, Cold Springs Harbor Press (1989); and Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Cold Spring Harbor, New York (1982), all of which are incorporated by reference herein. For example, conditions such as temperature.

5

10

15

20

25

10

15

20

25

30

concentration of components, hybridization and washing times, buffer components, and their pH and ionic strength may be varied.

Another aspect of the invention relates to a surface-modified electrode and its use in a bioelectrochemical process, in which electrons are transferred directly between an electrode and an electroactive biological material which is capable of accepting or donating one or more electrons. Such a bioelectrochemical process can be in either direction. In particular, the invention provides electrodes having their surface modified with oligonucleotide duplexes carrying an intercalative, redox-active moiety. The electrode can be of any material compatible with the surface modifier being adsorbed or bound thereon, including, but not limited to noble metals such as gold, silver, platinum, palladium, as well as carbon. The preferred material for the electrodes are gold and carbon.

The oligonucleotide duplex can be adsorbed onto the electrode in any convenient way. Preferably, the process of preparing such a modified electrode comprises adsorbing the oligonucleotide duplex which is derivatized at the 5'-end with a functionalized linker chains onto the electrode surface in one monolayer to obtain a uniform lawn. These linkers include, but are not limited to, thiol- or amineterminated chains. This process is generally understood by persons of ordinary skill in the art as and is relatively simple, reproducible and can easily be automated.

Furthermore, the density and composition of the monolayer is subject to variation depending on the selected assay. Methods of detecting single base-pair mismatches using intercalative, redox-active moieties require a densely packed monolayer to prevent the adsorbed intercalative, redox-active moieties from diffusing into the lawn. The method for detecting the presence or absence of a protein requires preferably an uneven monolayer comprised of duplexes of variable length to allow the protein to bind effectively to its recognition site along the duplex.

In addition, the present invention further relates to methods of creating a spatially addressable array of adsorbed duplexes. In a preferred embodiment, oligonucleotide duplexes of variable sequence composition that are derivatized at the 5'-end with a functionalized linker are deposited onto a multielectrode array. Subsequent treatment of these electrode-bound duplexes under denaturing conditions

yields a monolayer of single-stranded oligonucleotides, which can then be hybridized with a complementary oligonucleotide probe that potentially contains a mismatch. In another preferred embodiment, short oligonucleotide duplexes (5 to 10 base-pairs in length) that are derivatized on one end with a functionalized linker and contain single-stranded overhangs (5 to 10 nucleotides in length) of designed sequence composition at the opposite end are deposited onto a multielectrode array to generate a spatially addressable matrix. These electrode-bound duplexes can then be hybridized with single-stranded or double-stranded oligonucleotides that contain the complementary overhang.

Solid supports containing immobilized molecules have been extensively used in research, in clinical analyses and in the commercial production of foods and chemicals (see e.g., U.S. Pat. No. 5.153,166; Akashi, 1992). Immobilized nucleic acids are used in hybridization assays (Lamture, 1994) and immobilized proteins in radioimmuno or ELISA assays (see, U.S. Pat. No. 5,314,830). In addition, enzymes have been immobilized to facilitate their separation from product and to allow for their efficient and repetitive use. A number of important factors have to be considered in the development of an effective immobilization procedure. First, the procedure must minimize non-specific adsorption of molecules. Second, the procedure must maintain the functional integrity of the immobilized molecules. Third, the stability of the bond between the support and the immobilized molecule must be such to avoid leaching which would lead to reduced accuracy and sensitivity. Finally, the coupling procedure must be efficient enough to result in a support with a high capacity for the target molecules as well as be cost effective.

Another aspect of the invention relates to measuring the electrical current as a function of degree of hybridization of the oligonucleotide duplex adsorbed onto the electrode. When the intercalative, redox-active species is exposed to electrochemical or chemical energy, the electrical current may be continuously detected using techniques well known in the art. These include, but are not limited electronic methods, for example voltammetry or amperommetry, or optical methods, for example fluorescence or phosphoresence.

5

10

15

20

25

10

15

20

25

Generally, photoluminescence excitation and emission occur with electromagnetic radiation of between about 200 nanometers and about 900 nanometers in wavelength. Likewise, chemiluminescent and electrochemiluminescent emission generally occur with the emitted electromagnetic radiation being between about 200 nanometers and about 900 nanometers in wavelength. The potential at which the reduction or oxidation of the chemical moiety occurs depends upon its exact chemical structure as well as factors such as the pH of the solution and the nature of the electrode used. It is well known how to determine the optimal emission and excitation wavelengths in a photoluminescent system and the optimal potential and emission wavelength of an electrochemiluminescent and chemiluminescent system.

There are many methods for quantifying the amount of luminescent species present. The rate of energy input into the system can provide a measure of the luminescent species. Suitable measurements include, for example, measurements of electric current when the luminescent species is generated electrochemically, the rate of reductant or oxidant utilization when the luminescent species is generated chemically or the absorption of electromagnetic energy in photoluminescent techniques. In addition, the luminescent species can be detected by measuring the emitted electromagnetic radiation. All of these measurements can be made either as continuous, event-based measurements, or as cumulative methods which add the signal over a long period of time. Event-based measurements may be carried out with photomultiplier tubes, photodiodes or phototransistors to produce electric currents proportional in magnitude to the incident light intensity, or by using charge couple devices. Examples of cumulative methods are the integration of event-based data, and the use of photographic film to provide cumulative data directly.

The publications and other reference materials referred to herein describe the background of the invention and provide additional detail regarding its practice and are hereby incorporated by reference. For convenience, the reference materials are referenced and grouped in the appended bibliography.

The present invention is further described in the following examples. These examples are for illustrative purposes only, and are not to be construed as limiting the scope of the invention as set forth in the appended claims.

Examples

5

10

15

20

25

30

Materials. Phosphoramidite reagents (including the C_6S -S thiol modifier) were obtained from Glen Research. [γ - 32 P]dATP was obtained from NEN-DuPont. Potassium ferrocyanide (Fisher) was recrystallized from aqueous solution prior to use. Daunomycin was obtained from Fluka.

Synthesis of Derivatized Duplexes. Oligonucleotides immobilized on a controlled pore glass resin were treated in succession with carbonyldiimidazole and 1.6-diaminohexane (1g/10ml dioxane, 30 min/ea.) at the 5'-hydroxy terminus before cleavage from the resin (Wachter, 1986). After deprotection, the free amine was treated with 2-pyridylthiopropionic acid N-succinimide ester to produce a disulfide (Harrison, 1997). The sequences were purified by reverse-phase HPLC, converted to free thiols using dithiothreitol, and repurified before hybridization to their complements. Derivatized oligonucleotides were characterized by mass-assisted laser desorption ionization time-of-flight mass spectrometry and HPLC retention times. Duplexes were hybridized in deoxygenated 5 mM phosphate/50 mM NaCl (pH 7) by heating to 90 °C followed by slow cooling to room temperature. Unprotected duplexes were stored frozen under argon to prevention oxidation of the thiol.

Atomic Force Microscopy (AFM). All AFM images were collected using a MultiMode AFM running on the NanoScope IIIa controller (Digital Instruments, Santa Barbara, CA). A glass AFM chamber (Digital Instruments, Santa Barbara, CA) and a fluid volume of approximately 50 microliters were used for the experiments. Si₃N₄ cantilevers (spring constant, 0.06 N/m) with integrated, end-mounted oxide-sharpened Si₃N₄ probe tips were used. The applied vertical force of the AFM probe during imaging was minimized to beneath 100pN. Continually adjusting the cantilever deflection feedback setpoint compensated for thermal drifting of the cantilever and a consistent, minimum force was maintained AFM height calibrations were carried out on a NIST-traceable 180-nm height standard and then confirmed by measuring a single-atom step in the Au gold surface. The AFM images were recorded

10

15

20

25

30

in "Height" (or constant force) mode. Holes in the monolayer used to determine monolayer thicknesses were prepared by decreasing the scan size to approximately 100-150 nm, increasing the scan rate to 24-30 Hz, and increasing the vertical force by advancing the setpoint several units. After about one minute, the scan size, scan rate, and setpoint were returned to their previous values, and images featuring a bare gold square were captured. All images captured for height-contrast analysis were recorded at minimum vertical tip forces. This was accomplished by decreasing the set-point until the tip disengaged from the surface, then reintroducing it with the minimum force required to achieve a stable image. In several cases, the film height was also measured in tapping mode, and gave the same result as the contact-mode experiments.

Electrochemistry. Cyclic voltammetry (CV) was carried out on 0.02 cm² polycrystalline gold electrodes using a Bioanalytical Systems (BAS) Model CV-50W electrochemical analyzer at 20 ± 2 °C in 100 mM phosphate buffer (pH 7). A normal three-electrode configuration consisting of a modified gold-disk working electrode, a saturated calomel reference electrode (SCE, Fisher Scientific), and a platinum wire auxiliary electrode was used. The working compartment of the electrochemical cell was separated from the reference compartment by a modified Luggin capillary. Potentials are reported versus SCE. Heterogeneous electron-transfer rates were determined and analyzed by CV (Nahir, 1994; Weber, 1994; Tender, 1994).

Ellipsometry. Optical ellipsometry ($\lambda = 632.8 \text{ nm}$) was carried out on dried samples at 25 °C using a Gaertner Model L116C ellipsometer.

Example 1: Site-specific Incorporation of a Redox-Active Intercalator into a DNA Duplex.

The redox-active intercalator daunomycin (DM) (Arcamone, 1981) was incorporated into the DNA duplex to investigate charge transduction through these duplexes (Figure 1). DM undergoes a reversible reduction (Molinier-Jumel, 1978; Berg. 1981) within the potential window of the monolayers (Kelley, 1997a), and covalent adducts of intercalated DM crosslinked to the 2-amino group of guanine (Leng. 1996) have been crystallographically characterized within duplex DNA (Wang. 1991). Thus, a series of oligonucleotides primarily containing A-T or inosine (I)-C pairs were constructed with discrete guanine binding sites to which DM was

10

15

20

25

30

crosslinked. Preferably, thiol-terminated duplexes (0.1 mM) containing an adjacent pair of guanines were hybridized, incubated with 0.2 % formaldehyde and 0.2 mM DM in 5 mM phosphate, 50 mM NaCl, pH 7 for 1 h, and phenol extracted to remove excess DM.

Moving the guanine site along the duplex resulted in a systematic variation of the through-helix DM/gold separation, and allowed an investigation of the effect of distance on the dynamics of charge transport through the monolayers (Figure 1).

Example 2: Characterization of DNA Duplexes Modified with a Redox-Active Intercalator.

Modified duplexes were characterized by mass spectrometry, ultraviolet/visible absorption spectroscopy, and thermal denaturation experiments, all of which were consistent with a 1:1 DM-duplex stoichiometry. For example, the duplex SH-(CH₂)CONH(CH₂)6NHCO₂-5'ATCCTACTCATGGAC with its inosine complement modified with DM was analyzed by MALDI-TOF spectrometry. Massto-charge ratios (found/calc) of 5284/(5282) (DM + SH strand), 4541/(4540) (complement), and 4742/(4742) (SH strand) were detected. These values correspond to the calculated masses for fragments expected from this duplex. UV-visible absorption spectroscopy also revealed a 1:1 duplex/DM stoichiometry based upon comparison of the duplex absorbance at 260 nm ($\varepsilon = 14.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and the absorbance of intercalated DM at 480 nm ($\varepsilon = 5.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). In the presence of 100 mM phosphate, 100 mM MgCI₂, and at pH 7, thermal denaturation studies of 5 DM duplexes (monitored by absorbance at 260 nm) revealed melting temperatures of 48 and 50°C for the native and daunomycin-crosslinked duplexes, respectively. A similar melting profile was obtained by monitoring hypochromicity at 482 nm for the DM duplex.

Example 3: Preparation of Gold Electrodes Derivatized with DNA Duplexes.

Electrodes were conveniently prepared by modifying gold surfaces with 15 base-pair DNA duplexes derivatized at the 5' end with a thiol-terminated alkane chain. Bulk gold electrodes were polished successively with 0.3- and 0.5-μM alumina (Buhler), sonicated for 30 min, and etched in 1.0 M sulfuric acid. Au(111) surfaces

were prepared by vapor deposition onto mica or glass (Widrig, 1991; Zei, 1983). Electrodes vere then modified by incubation in 0.1 mM solutions of derivatized DNA duplexes in 5 mM phosphate/50 mM NaCl (pH 7) for 12 - 48 h at ambient temperature. Modified electrodes were rinsed in buffer prior to use.

Before deposition of the duplexes onto the gold surfaces, the presence of the free thiols was confirmed using a spectroscopic assay based on dithionitrobenzene (Riddles, 1979). Subsequently, the samples were deposited onto the gold surfaces for 12 - 24 h.

Electrochemical assays, radioactive tagging experiments, and atomic force microscopy (AFM) (Kelley, 1997a, 1997b) all indicate that the oligonucleotides form densely packed monolayers oriented in an upright position with respect to the gold surface.

Example 4: Characterization of Modified DNA Duplexes Monolayers on Gold Surfaces

The DM-modified duplexes readily formed self-assembled monolayers on gold. AFM studies of modified films reveal densely packed monolayers with heights greater than 45 Å at open circuit. More specific, AFM studies were carried out under electrochemical control, and revealed that the DNA films undergo a potential-dependent change in structure. At open circuit, the monolayer film height is 45(3) Å. Based on the anisotropic dimensions of the 15-base pair duplexes (20 Å in diameter vs. 60 Å in length), this thickness indicates that the helical axis is oriented ~ 45" from the gold surface. At applied voltages negative of the potential of zero charge, film thickness of ~60 Å are observed; more positive potential cause a drop in the film height to a limiting value of 20 Å at low surface coverages.

Based on the crossectional area of DNA ($\sim 3 \text{ nm}^2$) and the geometrical area of the gold electrodes (0.02 cm²), the maximum surface coverage of DNA was calculated as $\sim 6 \times 10^{-11} \text{ mol/cm}^2$. Coulometry at electrodes modified with duplexes containing crosslinked DM revealed a DM surface coverage of 7.5(7) x $10^{-11} \text{ mol/cm}^2$, indicating that the surface is densely packed with the modified duplexes. The DM value appeared to exceed slightly the theoretical Γ for DNA, and likely resulted from additional electrode surface roughness.

1

5

10

15

20

25

10

15

20

25

30

To assess routinely the surface coverage of DM-derivatized DNA on gold, the electrochemical response of Fe(CN)₆ ⁴⁻ (2 mM) was monitored. This negatively charged ion is repelled from the modified-electrode surface by the polyanionic DNA, and exhibits essentially no response when the surface is well covered. While not a direct measure of surface coverage, this technique allowed the convenient assay of individual electrodes for adequate modification.

Cyclic voltammograms of these surfaces showed the reversible reduction of DM at -0.65 V versus SCE (Molinier-Jumel, 1978; Berg, 1981). These films were extremely stable and exhibited responses characteristic of surface-bound species (e.g., linear plots of peak current versus scan rate) (Bard, 1980).

Example 5: Measurement of Electrochemical Response of a Redox-Active Intercalator Crosslinked to a *Fully Base-Paired* DNA Duplex on a Gold Surface.

Integration of the electrochemical response yielded a surface coverage (Γ) of electroactive daunomycin of 7.5(7) x 10⁻¹¹ mol/cm², a value in good agreement with the coverages of 15-base pair duplexes previously measured via ³²P labeling (Kelley, 1997a). However, significant fluctuations in the surface coverages of DM-modified duplexes were observed. Therefore, only electrodes which exhibited both large integrated currents for the reduction of crosslinked DM *and* an attenuated responses for the oxidation of ferrocyanide in solution were studied.

Given the 1:1 stoichiometry of crosslinked DM to DNA, the observed data indicated that all of the bound DM was electrochemically reduced. Doping these films with increasing percentages of DM-free duplexes resulted in a linear decrease in the observed electrochemical signals (as determined from coulometric assays), consistent with each of the bound intercalators being electrochemically active.

Remarkably, efficient reduction of DM was observed regardless of its position along the 15-base-pair sequence as illustrated in Figure 2. Based on molecular modeling, the DM/gold separations span ~ 25 Å. The through-helix DM-electrode separation is > 10 Å for DM bound at the end of the duplex closest to the electrode (Figure 2A), and the DM-electrode separation is > 35 Å (Figure 2B) for DM crosslinked to the end of the duplex farthest from the electrode. The surface coverage

of electroactive daunomycin for these 15 base-pair duplexes as measured by integrating the currents within the illustrated voltammograms were 0.65×10^{-10} mol/cm² and 0.80×10^{-10} mol/cm², respectively. The DM:DNA stoichiometry for these same samples, measured by absorption spectroscopy were 0.9:1 and 1.1:1, respectively. Thus, the charge did not depend on distance, but did reflect the yield of crosslinking.

Example 6: Measurement of Electrochemical Response of a Redox-Active Intercalator Crosslinked to a *Mismatch-Containing* DNA Duplex on a Gold Surface.

Electrochemical responses of a redox-active intercalator crosslinked to a *mismatch-containing* DNA duplex on a gold surface were measured to determine whether these observed rates were a result of direct contact between the redox-active cofactor and the electrode surface (which has previously been shown to yield apparently distance-independent heterogeneous electron transfer (Feng, 1995, 1997)). A single site within the 15-base-pair duplex was mutated to produce a CA mismatch (known to cause local disruptions in the DNA base stack (Patel, 1984; Aboul-ela, 1985) between the intercalated DM and the electrode surface. Figure 3 illustrates that such a simple change virtually eliminated the electrochemical response.

The coulometry of DM at electrodes modified with CA-containing duplexes varied to some degree as a function of the surface coverage. At high surface coverages (as determined by the ferrocyanide assay), essentially no signal was observed with the mismatched duplexes. However, at more moderate surface coverages, small signals corresponding to the reduction of DM were found. These typically did not exceed 30 % of the signals found for the TA duplexes. The morphology of partial DNA monolayers is unknown.

Significantly, sequences in which the positions of the DM and CA mismatch were reversed (such that the mismatch was located above the DM relative to the gold) showed no diminution in the electrochemical response. AFM images of the CA-mutated sequences were identical to those of the TA analogs (monolayer thicknesses of $\sim 40\text{Å}$ at open circuit), revealing that the bulk structure of the DNA films was not significantly altered by the presence of a mismatch. Moreover, the oxidation of

10

15

20

25

10

15

20

25

30

ferrocyanide was similarly attenuated at both surfaces. Expected masses for DM-crosslinked DNA duplexes (accounting for the single base change) were measured by mass spectrometry, and spectrophotometric assays revealed that the extent of crosslinking was identical in both fully paired and mismatched sequences.

The exquisite sensitivity of the electrochemistry of DM to intervening lesions in the base stack provides therefore the basis for an exceptionally versatile DNA-mismatch sensor.

Example 7: Analysis of the Electrochemical Behavior of Fully Base-Paired or Mismatch-Containing DNA Duplexes Containing Non-Crosslinked Intercalators.

A practical method to detect mismatches utilizes a system based on non-crosslinked, intercalative, redox-active species. The electrochemistry of DM non-covalently intercalated into DNA-modified films was studied in order to develop a general approach to test heterogeneous sequences that may possess more than one guanine-binding site. Coulometric titrations confirmed that DM strongly binds to surfaces modified with fully base-paired duplexes, and yielded affinity constants very similar to those determined for homogeneous solutions (Arcamone, 1981; Molinier-Jumal, 1978; Berg, 1981). At bulk DM concentrations ≥1 μM, the modified electrodes were saturated with intercalator, and hold approximately one intercalator per surface-confined duplex. Furthermore, intercalators non-covalently bound to these films exhibited electrochemical properties quite similar to those described for crosslinked DM, with the exception that the binding was reversible, i.e. in pure buffer solutions, decreasing voltammetric signals were observed until total dissociation was evident.

In accord with the studies of covalently bound DM, incorporation of a single CA mismatch into these duplexes dramatically decreased the electrochemical response (Table 1). The magnitude of this mismatch effect depended strongly on the location of the CA base step along the sequence: when the mutation was buried deep within the monolayer, the measured charge drops by a factor-of 3.5(5) (relative to the Watson-Crick duplex), but by only 2.3(4) when it was located near the solvent-exposed terminus. These observations were consistent with DM occupying sites near

10

15

20

25

30



the top of the densely packed monolayer, as suggested in earlier studies of methylene blue bound to these same surfaces (Kelley, 1997b). The intensity of the electrochemical signals therefore not only reports the presence of the mismatch but also may describe the location of the disruption.

34

In addition, lateral charge diffusion within these monolayers was analyzed. For example, a series of fully base-paired films (sequence: SH-5'AGTACAGTCATCGCG) doped with increasing fractions of CA-mismatched helices were prepared (the mismatch was localized at the base step denoted by the bold C in the above sequence.) The coulometric response of DM non-covalently bound to these surfaces was strongly dependent on the film composition such that the electrochemical signals decreased linearly with increasing percentages of mutated duplexes. As there is no measurable difference in the affinities of DM toward TA-versus CA-containing films, this linear response indicated that the electroinactive intercalators (presumably those molecules bound to mutated helices) are not reduced by lateral charge transfer from the electroactive species. This result further supports a through-helix pathway for charge transduction, as intermolecular interactions between intercalators bound to different duplexes in the film evidently do not mediate efficient electron transfer.

Example 8: Analysis of Mutation Dependence of Electrochemical Response.

To explore the scope of this mismatch detection strategy, the charge (Q_c) for DM at DNA-modified electrodes containing different single-base mismatches was analyzed (Figure 4). The seven different mismatched duplexes were obtained by hybridization of the thiol-modified sequence, SH- 5 'AGTACAGTCATCGCG, with the following seven different complements (the mismatch is indicated in bold, and the specific basepair and the melting temperature of the duplex is given in parentheses): 5 'CGCGATGACTGTACT (TA, $T_m = 68$ °C), 5 'CGCGACGACTGTACT (CA, $T_m = 56$ °C), 5 'CGCGATGACTGTACT (TT, $T_m = 57$ °C), 5 'CGCGATCACTGTACT (CC, $T_m = 56$ °C), 5 'CGCGATGGCTGTACT (GT, $T_m = 62$ °C), 5 'CGCGATGAATGTACT (GA, $T_m = 60$ °C), 5 'CGCGATGCCTGTACT (CT, $T_m = 58$ °C). The charges were then calculated by integrating background-subtracted cyclic voltammograms. The

10

15

20

obtained values were based on > 5 trials, and the results were comparable for experiments run side-by-side or utilizing different sample preparations. The melting temperatures of the oligomers in solution were measured by monitoring duplex hypochromicity at 260 nm using samples that contained 10 μM duplex, 100 mM MgCl₂, and 100 mM phosphate at pH 7.

Coulometric analysis confirmed that the attenuation of the characteristic DM response was strongly dependent upon the identity of the mutation. In general, pyrimidine-pyrimidine and purine-pyrimidine mismatches caused marked decreases in the electrochemical signals; the one purine-purine pair studied (a GA mismatch, which is notoriously well-stacked within duplex DNA (Patel, 1984; Aboul-ela, 1985)) did not show a measurable effect. Suprisingly, a significant decrease was caused by a GT pair, which is also not highly disruptive to the helix. This wobble base pair, although thermodynamically stable, appears to mediate electron transfer poorly.

Figure 4 illustrates that across a very narrow range of duplex thermal stabilities, large differences in the electrochemical response were observed. Overall, the electrochemical properties of films containing the different mismatches correlated with the degree of disruption to base stacking with the individual duplexes. These results underscore the sensitivity of this electrochemical assay to base stacking within DNA, and demonstrate the viability of detecting mismatches based upon charge transduction through thin films.

Example 9: Analysis of Sequence Dependence of Mismatch Detection Assay.

A single CA mismatch was incorporated into three different DNA duplexes to test for the sequence dependence of the assay. The duplexes featured varying percentages of GC content, representing a wide range of duplex stabilities. The 25 melting temperatures for these duplexes, as determined by thermal denaturation measurements obtained by monitoring hypochromicity at 260 nm in duplex solutions containing 10 µM duplex, 100 mM phosphate, and 100 mM MgCl₂ were: (SH-5'-ATATAATATGGAT): TA = 47 °C. CA = 32 °C; (SH- 5 -AGTACAGTCATCGCG): TA = 68 °C, CA = 56 °C; (SH- 5'-

30 GGCGCCCGGCGCGG): GC = 82 °C, CA = 69 °C. The charge was quantitated from integrating background-subtracted cyclic voltammograms obtained at $\upsilon=100$ mV/s and was corrected for electrode area. As illustrated in Figure 5, the characteristic drop in coulometric signals for DNA duplexes containing a single CA mismatch compared to fully base-paired DNA films was essentially invariant across AT-rich to GC-rich sequences. This sequence-independent response is not achievable using traditional mismatch detection assays based upon differential hybridization.

Example 10: Analysis of Electrochemical Response During Repeated Cycles.

To extend this methodology to single-stranded targets, techniques for *in situ* hybridization were developed. Thiol-modified duplexes were deposited on the gold surface, heat denatured, thoroughly rinsed, then rehybridized with the desired target by incubation in ≥ 50 pmol of single-stranded oligonucleotide. The electrochemical properties of the resulting surfaces were identical to those described above, suggesting the suitability of this system for genomic testing.

For example, a 15-base-pair oligonucleotide. ⁵ AGTACAGTCATCGCG, which was derivatized with a thiol-terminated linker, was hybridized both to its native complement and to a mutated complement (at the site underlined in the sequence), generating a fully base-paired duplex and a CA mismatch-containing duplex. respectively (Figure 6). These duplexes were deposited on separate electrodes and the electrochemical responses of DM non-covalently bound to these duplexes were measured using cyclic voltammetry ($\nu = 100$ mV/s, 1.0 μ M DM). Figure 6 illustrates that DM exhibited electrochemical responses characteristic of fully base-paired and CA-mutated films, respectively. The surfaces were then denatured by immersing the electrodes in 90 °C pure buffer for 2 min to yield single-stranded monolayers of identical sequence. Cyclic voltammetry of DM at these electrodes now revealed nearly identical responses, with the reduction appearing highly irreversible, broadened, and becoming smaller as a function of increasing scans. Importantly, the electrode that initially possessed the CA mismatch displayed a large signal (for the first scan) after denaturation, while the reverse was true for the corresponding TA analog. New duplexes were formed by incubating the electrodes with 100 pmol of the opposite complement in the presence of buffered 100 mM MgCl₂ such that the

5

10

15

20

25

WO 99/51778

complements were traded (TA \rightarrow CA, CA \rightarrow TA), and the electrochemistry at the duplex-modified films again showed the characteristic behavior expected for fully base-paired and CA-mutated films. Finally, the electrodes were again heated to denature the duplexes and quantitation of the response showed again the characteristics for single-stranded oligonucleotides. Thus, electrodes can be cycled through this sequence of events repeatedly, indicating a practical means to detect point mutations within natural DNAs.

37

Example 11: Detection of Genetic Mutations Within a Specific Region of the p53 Gene Using Direct Current Measurement of Thiol-Modified Duplexes on Gold Surfaces.

A specific embodiment utilizes a gold-microelectrode array with approximately thirty addressable sites. A different 20-base pair duplex derivatized with a hexylthiol linker is attached to each of these sites by deposition form a concentrated duplex solution overnight. The sequences are chosen to correspond to the 600-base pair region within exons 5 through 8 of the p53 gene where most of the cancer-related mutations are found. The array is immersed in aqueous solution at 90 °C for 60 seconds to denature the immobilized duplexes and remove the complementary strands. The human sample containing the p53 gene is fragmented either before or after amplification. A solution containing the fragmented genomic single-stranded DNA is deposited on the array for one hour to allow hybridization to occur. Then, in the presence of a 1.0 μ M DM solution, the charge passed at each of the electrodes is measured, and the response for each sequence is compared to that obtained from the wild-type (i.e. fully base-paired) sequences. Electrodes with attenuated signals correspond to mutated subsequences, while those which exhibited the expected charge are classified as unmutated.

Example 12: Detection of Mutations Using Electrocatalytic Currents Generated at DNA-Modified Surfaces.

The signals corresponding to mismatched and fully-paired sequences can be more highly differentiated by monitoring catalytic currents at DNA-modified surfaces. Electrons can be shuttled through the immobilized duplexes to redox-active intercalators localized on the solvent-exposed periphery of the monolayer, and then

5

10

15

20

25

10

15

20

25

30

negatively-charged solution-borne species (which are electrostatically prohibited from the interior of the monolayer) are catalytically reduced by the intercalating mediators. Since the catalytic reaction essentially amplifies the signal corresponding to the intercalator, the attenuation of this response in the presence of the mismatch is significantly more pronounced. In a specific embodiment, the sequence SH-⁵AGTACAGTCATCGCG was deposited on an electrode both hybridized with a fully base-paired complement, and with a complement containing a CA mismatch (the position of the mismatch is denoted in bold). These duplexes were immersed in a solution containing 1.0 µM methylene blue and 1 mM ferricyanide. In the presence of either of these reagents alone, only small direct currents were measured. However, in the presence of a mixture of the intercalator and the negatively charged probe, pronounced currents were measured corresponding to the electrocatalytic reduction of ferricyanide by methylene blue. The amount of current observed for the TA and CA containing films differ dramatically; using electrocatalysis, the mismatched duplex can be differentiated from the fully base-paired duplexes by a factor of approximately 100. Moreover, as illustrated in Figure 8, the peak potentials for the TA and CA duplexes are significantly separated, allowing the presence of the mismatch to be detected potentiometrically. This approach therefore represents an extremely sensitive means to detect genetic mutations electrochemically.

Example 13: Increased Sampling Time Using Electrocatalytic Currents Generated at DNA-Modified Surfaces Increases Sensitivity.

Because the charge transport-based assay features a catalytic reaction whose rate depends on the degree of complementarity within the individual duplexes, the measured charge resulting from the reduction of methylene blue at TA versus CA-containing films increases disproportionately with longer integration times (Figure 9). Using $0.5~\mu\text{M}$ methylene blue and 2.0~mM ferricyanide, 10-second potential steps to -350 mV gave faradaic charges of 36 and 6 μC respectively. Increased sampling times continue to increase the differentiation of signals obtained with mismatched versus paired complements. These results highlight the versatility of electrochemical

10

15

20

25

30

detection methods which are also more amenable to the portability required for a practical device.

Example 14: Detection of Genetic Mutations Within a Specific Region of the p53 Gene Using Electrocatalytic Current Measurement of Thiol-Modified Duplexes on Gold Surfaces.

Another specific embodiment involves detecting the mutations within the p53 gene using electrocatalysis. A different 20-base pair duplex derivatized with a hexylthiol linker is attached to each of approximately thirty addressable sites of a gold-microelectrode array by deposition form a concentrated duplex solution overnight. The sequences are chosen to correspond to the 600-base pair region within exons 5 through 8 of the p53 gene where most of the cancer-related mutations are found. The array is immersed in aqueous solution at 90 °C for 60 seconds to denature the immobilized duplexes and remove the complementary strands. The human sample containing the p53 gene is fragmented either before at after amplification. A solution containing the fragmented genomic single-stranded LNA is deposited on the array for one hour to allow hybridization to occur. The array is rinsed and submerged in a solution containing 1.0 µM methylene blue and 1.0 mM ferricyanide. The pronounced currents that are observed result from the electrocatalytic reduction of the solution-borne ferricyanide by methylene blue adsorbed at the solvent-exposed duplex sites. These catalytic currents are measured for each addressable electrode and compared with those obtained with the wild-type sequence to detect potential sites of mutations.

Example 15: Detection of Genetic Mutations Within a Gene of Interest Using Direct or Electrocatalytic Current Measurement of Amine-Modified Duplexes on Carbon Surfaces.

Another embodiment utilizes a carbon electrode. The electrode is oxidized at $\pm 1.5 \text{ V}$ (vs. Ag/AgCl) in the presence of $\text{K}_2\text{Cr}_2\text{O}_7$ and HNO_3 , and treated with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS). Duplexes corresponding to mutated sequences of a specific gene of interest are derivatized with a hexylamine linker and applied to the electrode surface. The device is immersed in aqueous solution at 90 °C for 60 seconds

to, generate a single-stranded monolayer, and the fragmented genomic DNA sample is hybridized to the immobilized probes at room temperature for 1 hour. The detection of mutations is accomplished by (i) measuring direct currents in the presence of 1.0 μ M daunomycin solution, or (ii) by measuring catalytic currents in the presence of 1.0 μ M methylene blue and 1.0 mM ferricyanide. Charges passed at each electrode are measured, and the response for each sequence is compared to that obtained with wild-type, i.e. fully base-paired, sequences. Attenuated signals correspond to mutated subsequences, while those which exhibit no change in current are classified unmutated.

Although the invention has been described with reference to particular applications, the principles involved may be used in other applications which will be apparent to those skilled in the art. The invention is accordingly to be limited only by the scope of the claims which follow.

Table I. Electrochemical Detection of Single-Base Mismatches^a

	Q _{c(int)} (nC) ^b	T _m (°C)°
SH- ⁵ AGTACAGTCATCGCG TCATGTCAGTAGCGC	165(37)	89
SH-AGTACAGTCATCGCG TCATGTCAGCAGCGC	56(15)	56
SH-AGTACAGTCATCGCG TCATGTCTGTAGCGC	95(18)	57
SH ⁻² AGTACAGT C ATCGCG TCATGTCA C TAGCGC	51(23)	56
SH-AGTACAG T CATCGCG TCATGTC G GTAGCGC	49(30)	62
SH-AGTACAGTCATCGCG TCATGTAAGTAGCGC	153(38)	09
SH- ⁵ 'AGTACAG T CATCGCG TCATGTC C GTAGCGC	93(17)	58

higher charges (> 1 DM/duplex), and decreased atternations in the presence of mismatches. Pintegrated background-subtracted cathodic different sample preparation as long as electrodes exhibited high surface coverages. Electrodes with lower surface coverages yielded phosphale buffer, pH 7). Values are based on > 5 trials each, and results were comparable for experiments run side-by side, or from charge. ^cMeasured **by mo**nitoring duplex hypochromicity at 260 nm. Samples contained 10 μM duplex, 100 mM MgCl₂, 100 mM *Based on cyclic voltammograms measured for 1.0 µM daunomych noncovalently bound to duplex-modified electrodes (0.1 M phosphate, pH 7.

8. REFERENCES

- Aboul-ela, F., Koh, D., Tinoco, I., Martin, F.H. (1985) Nucl. Acids Res. 13: 4811.
- Akashi et al. (1992) Bioconjugate Chem. 3: 363.
- Alivisatos, A.P., Johnsson, K.P., Peng, X.G., Wilson, T.E., Loweth, C.J., Bruchez, M.P., Schultz, P.G. (1996) *Nature* 382: 609.
 - Arcamone, F. Doxorubicin: Anticancer Antibiotics, Academic Press, New York (1981).
 - Arkin, M.R., Stemp, E.D.A., Barton, J.K. (1997) Chem. and Biol. 4: 389.
- Bard A.J. and Faulkner, L.R. *Electrochemical Methods*, Wiley and Sons, New York 10 (1980).
 - Beratan, D.N., Priyadarshy, S., Risser S.M. (1997) Chem. and Biol. 4: 3.
 - Berg, H., Horn, G., Luthardt, U. (1981) Biolectrochem. Bioenerg. 8: 537.
 - Bos (1989) Canc. Res. 49: 4682.
 - Brun A.M. and Harriman A. (1992) J. Am. Chem. Soc. 114: 3656.
- 15 Carter, M. T. et al. (1989) J. Am. Chem. Soc. 111: 8901.
 - Carter, M. T. et al. (1990) Biocnjugate Chem. 1: 257.
 - Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhard, D.J., Morris, M.S., Fodor, S.P.A. (1996) *Science* 274: 610-614.
 - Dandliker, P.J., Homlin, R.E., Barton, J.K. (1997) Science 275: 1465.
- Drmanac, R., Drmanac, S., Strezoska, Z., Paunesku, T., Labat, I., Zeremski, M., Snoddy, J., Funkhouser, W. K., Koop, B., Hood, L. et al. (1993) Science 260: 1649-1652.
 - Elghanian, R., Storhoff, J.J., Mucic, R.C., Letsinger, R.L., Mirkin, C.A. (1997) Science 277: 1078.
- 25 Eng, C. and Vijg, J. (1997) *Nat. Biotech.* **15:** 422.
 - Feng, Z.Q., Imabayashi, S., Kakiuchi, T., Niki, K. (1995) J. Electroanal. Chem. 394: 149.
 - Feng, Z.Q., Imabayashi, S., Kakiuchi, T., Niki, K. (1997) J. Chem. Soc. Faraday Trans. 93: 1367.
- 30 Hacia, J. G., Brody, L. C., Chee, M. S., Fodor, S. P., Collins, F. S. (1996) Nat. Genet. 14: 441-447.

- Hall, D.B., Homlin, R.E., Barton, J.K. (1996) Nature 382: 731.
- Hall D.B. and Barton, J.K. (1997) J. Am. Chem. Soc. 119: 5045.
- Harrison J.G. and Balasubramanian, S. (1997) Bioorg. Med. Chem. Lett. 7: 1041.
- Hashimoto, K. et al. (1994a) Anal. Chem. 66: 3830.
- Hashimoto, K. et al. (1994b) Anal. Chim. Acta 286: 219.
 - Herne, T. and Tarlov, M.J. (1997) J. Am. Chem. Soc. 119: 8916.
 - Homlin, R.E., Dandliker, P.J., Barton, J.K. (1997) Angew. Chem. Int. Ed., in press.
 - Innis et al. (1990) PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego.
- Jackson, B. A. and Barton, J. K. (1997) J. Am. Chem. Soc. 119: 12986-12987. 10
 - Johnston, D. H. et al. (1995) J. Am. Chem. Soc. 117: 8933.
 - Kelley, S.O., Barton, J.K., Jackson, N.M., Hill, M.G. (1997a) Bioconj. Chem. 8: 31.
 - Kelley, S.O., Barton, J.K., Jackson, N.M., McPherson, L., Potter, A., Spain, E.M., Allen, M.J., Hill, M.G., submitted (1997b)
- Kelley, S.O., Homlin, R.E., Stemp, E.D.A., Barton, J.K. (1997c) J. Am. Chem. Soc. 15 119: 9861.
 - Kelley S.O. and Barton, J.K. submitted (1997d).
 - Kelly, J. M. et al. (1986) In: Electrochemistry, Sensors, and Analysis, eds. Smyth, M. R. and Vos, J. G., Elsevier, Amsterdam, pp. 205.
- Kobayashi, H. Horton, E.L., Brown, (1996) Nat. Biotech. 14: 1675. 20
 - Korriyoussoufi, H., Garnier, F., Srivastava, P., Godillot, A., Yassar, (1997) J. Am. Chem. Soc. 119:7388.
 - Lamture et al. (1994) Nucl. Acids Res. 22: 2121.
 - Landegren et. al. (1988) Science 241: 1077.
- Leng, F., Savkur, R., Fokt, I., Prewloka, T., Priebe, W., Chaires, J.B. (1996) J. Am. 25 Chem. Soc. 118:4732.
 - Lewis, F.D., Wu, T., Zhang, Y., Lestsinger, R.L., Greenfield, S.R., Wasielewski M.R. (1997) Science 277: 673.
- Lin, S.V.-Y., Motesharei, K., Dancil, K.-P.S., Sailor, M.J., Ghadiri, M.R. (1997) 30 Science **278**: 840.

Lipshutz, R. J., Morris, D., Chee, M., Hubbell, E., Kozal, M. J., Shah, N., Shen, N., Yang, R., Fodor, S. P. (1995) *Biotechniques* 19: 442-447.

Maeda, M. et al. (1992) Anal. Sci. 8: 83.

Maeda, M. et al. (1994) Chem. Lett. 1805.

Marcus R.A. and Sutin N. (1985) Biochim. Biophys. Acta 811: 265.

Maxam and Gilbert (1980) Meth. Enz. 65: 499-560.

Meade, T. J. and Kayyem, J.F. (1995) Angew. Chem. Int. Ed. Engl. 34: 352.

Millan, K. M. and Mikkelsen, S. R. (1993) Anal. Chem. 65: 2317.

Molinier-Jumel, C., Malfoy, B., Reynaud, J.A., Aubel-Sadron, G (1978). Biochem. Biophys. Res. Comm. 84: 441.

Murphy, C.J., Arkin; M.A., Jenkins, Y., Ghatlia, N.D., Bossman, S., Turro, N.J.,

Barton, J.K. (1993) Science **262**: 1025.

Nahir, T.M., Clark, R.A., Bowden, E.F. (1994) Anal. Chem. 66: 2595.

Netzel T.L. J. (1997) Chem. Educ. 74: 646.

15 Okahata, J. et al. (1992) J. Am. Chem. Soc. 114: 8300.

Patel, D.J., Kozlowski, S.A., Ikuta, S., Itakura, K. (1984) FASEB 11: 2664.

Plambeck, J. and Lown, J. W. (1984) J. Electrochem. Soc. 131: 2556.

Priyadarshy, S., Risser, S.M., Beratan, D.N. (1996) J. Phys. Chem. 100: 17678.

Riddles, P.W., Blakeley, R.L., Zerner, B. (1979) Anal. Biochem, 94:75.

20 Rodriguez, M. and Bard, A. J. (1990) Anal. Chem. 62: 2658.

Saiki et al. (1985) Science 230: 1350.

Sanger et al. (1977) Proc. Natl. Acad. Sci. 74: 5463.

Scharf (1986) Science 233: 1076.

Skogerboe, K. J. (1993) Anal. Chem. 65: 416R.

25 Southern, E. M. (1996) Trends in Genetics 12: 110.

Szent-Györgyi, A. (1941) Nature 148: 157.

Tender, L., Carter, M.T., Murray, R.W. (1994) Anal. Chem. 66: 3173.

Wachter, L., Jablonski, J.A., Ramachandran, K.L. (1986) Nucl. Acids Res. 14: 7985.

Wang, A.H.-J., Gao, Y.-G., Liaw, Y.C., Li, Y.K. (1991) Biochemistry 30: 3812.

Warman, J.M., de Haas, M.P., Rupprecht, A. (1996) Chem. Phys. Lett. 249: 319.
 Weber, K., Creager, S.E. (1994) Anal. Chem. 66: 3164.

WO 99/51778 PCT/US99/07650

45

Welch, T. W. et al. (1995) J. Phys. Chem. 99 11757.

Widrig, C.A., Alves, C.A., Porter, M. D. (1991) J. Am. Chem. Soc. 113: 2805.

Wu, D. Y. et al. (1989a). Genomics 4: 560-569.

Xu, X.-H. et al. (1994) J. Am. Chem. Soc. 116: 8386.

5 Xu, X.-H. and Bard, A. J. (1995) J. Am. Chem. Soc. 117: 2627.

Zei, M.S., Nakai, Y., Lehmpfuhl, G., Kolb, D.M. (1983) *J. Electroanal. Chem.* **150**: 201.

WHAT IS CLAIMED IS:

- 1 1. A composition comprising a first single stranded nucleic acid
- 2 hybridized to a second single stranded nucleic acid forming a complex, said complex
- 3 containing one or more electron donor moieties and one or more electron acceptor
- 4 moieties combined therewith, wherein one of the electron donor or acceptor moieties
- 5 is an electrode or an addressable multielectrode array and the other electron donor or
- 6 acceptor moiety is an intercalative, redox-active moiety.
- 1 2. A composition according to claim 1, wherein said addressable
- 2 multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to
- 3 10 base-pairs in length, deposited onto said array, wherein each of said
- 4 oligonucleotide duplexes is derivatized on one end with a functionalized linker and on
- 5 the opposite end with a single-stranded overhang of distinct sequence composition.
- ϵ and wherein said single-stranded overhang is 5 to 10 nucleotides in length and can be
- 7 the same or different.
- 1 3. A composition according to claim 1, wherein said intercalative, redox-
- 2 active moiety is either noncovalently adsorbed or crosslinked to said oligonucleotide
- 3 duplex.
- 1 4. A composition according to claim 1, wherein said intercalative, redox-
- 2 active moiety is an intercalator.
- 1 5. A composition according to claim 1, wherein said intercalative, redox-
- 2 active moiety is an intercalator selected from the group consisting of phenanthridines,
- 3 phenothiazines, phenazines, acridines, and anthraquinones.
- 1 6. A composition according to claim 1, wherein said intercalative, redox-
- 2 active moiety is daunomycin.
- 1 7. A composition according to claim 1 wherein said intercalative, redox-
- 2 active moiety is part of a protein.
- 1 8. A composition according to claim 1 wherein said intercalative, redox-
- 2 active moiety is mut Y.

	9.	A sensor comprising:
2		(a) an electrode or an addressable multielectrode array;
3		(b) a polynucleotide duplex deposited onto at least one surface of
1	said electrode	or addressable multielectrode array, wherein said polynucleotide duplex
5	is comprised of	of a first single-stranded nucleic acid hybridized to a second single-
6	stranded nucle	eic acid forming a complex which possibly contains one or more
7	mismatches o	r one or more base-stacking perturbations; and
8		(c) an intercalative, redox-active moiety contacting said
9	polynucleotid	le duplex.
1	10.	A sensor according to claim 9, wherein said intercalative, redox-active
2	moiety is eith	ner noncovalently adsorbed or crosslinked to said polynucleotide duplex.
1	11.	A sensor according to claim 9, wherein said intercalative, redox-active
2	moiety is an	intercalator.
1	12.	A sensor according to claim 9, wherein said intercalative, redox-active
2	moiety is an	intercalator selected from the group consisting of phenanthridines,
3	phenothiazin	es, phenazines, acridines, and anthraquinones.
ı	13.	A sensor according to claim 9, wherein said intercalative, redox-active
2	moiety is da	unomycin.
1	14.	A sensor according to claim 9, wherein said intercalative, redox-active
2	moiety is pa	rt of a protein.
1	15.	A sensor according to claim 9, wherein said intercalative, redox-active
2	moiety is m	ut Y.
1	16.	A sensor according to claim 9, wherein said electrode or addressable
2	multielectro	ode array is gold.
1	17.	A sensor according to claim 9, wherein said electrode or addressable
2	multielectro	ode array is carbon.
1	18.	A sensor according to claim 9, wherein one of said nucleic acids is
2	derivatized	with a functionalized linker.

1	19.	A sense	or according to claim 18, wherein said functionalized linker is
2	comprised of	5 to 20 c	5 bonds.
1	20.	A sens	or according to claim 18, wherein said functionalized linker is
2	thiol-terminate	ed.	
1	21.	A sens	or according to claim 18, wherein said functionalized linker is
2	amine-termina	ated.	
1	22.	A sens	or according to claim 9, wherein said addressable multielectrode
2	array is comp	rised of	oligonucleotide duplexes of 5 to 10 base-pairs in length,
3	•		ray, wherein each of said oligonucleotide duplexes is derivatized
4			ctionalized linker and on the opposite end with a first single-
5	stranded over	hang of	known sequence composition, and wherein one of said nucleic
6	acids has a se	cond sin	igle-stranded overhang that is complementary to said first single-
7	stranded over	hang on	said addressable multielectrode array.
1	23.	A sens	sor comprising:
2		(a)	an electrode or an addressable multielectrode array comprised
3	of gold;		
4		(b)	one or more oligonucleotide duplexes deposited onto at least
5	one surface o	f said el	ectrode or addressable multielectrode array, wherein said
6	oligonucleoti	de duple	exes are comprised of a first single-stranded nucleic acid
7	hybridized to	a secon	d single-stranded nucleic acid, and wherein one of said nucleic
8	acids is deriv	atized w	vith a thiol-terminated linker comprised of 5 to 20 σ-bonds; and
9		(c)	an intercalative moiety site-specifically adsorbed to said
10	oligonucleoti	ide duple	exes, wherein said intercalative moiety is daunomycin, and
11	wherein said	oligonu	cleotide duplexes provide electrical contact between the electrode
12	or addressab	le multie	electrode array and daunomycin.
1	24.	A sen	sor comprising:
2		(a)	an electrode or an addressable multielectrode array comprised
3	of carbon;		

4	(b) one or more oligonucleotide duplexes deposited onto at least
5	one surface of said electrode or addressable multielectrode array, wherein said
6	oligonucleotide duplexes are comprised of a first single-stranded nucleic acid
7	hybridized to a second single-stranded nucleic acid, and wherein one of said nucleic
8	acids is derivatized with an amine-terminated linker comprised of 5 to 20 σ -bonds;
9	and
10	(c) an intercalative moiety site-specifically adsorbed to said
11	oligonucleotide duplexes, wherein said intercalative moiety is daunomycin, and
12	wherein said oligonucleotide duplexes provide electrical contact between the electrode
13	or addressable multielectrode array and daunomycin.
l	25. A sensor comprising:
2	(a) an electrode or addressable multielectrode array, wherein said
3	addressable multielectrode array is comprised of a monolayer of oligonucleotide
4	duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of
5	said oligonucleotide duplexes is derivatized on one end with a functionalized linker
6	and on the opposite end with a first single-stranded overhang of known sequence
7	composition, and wherein said first single-stranded overhang can be the same or
8	different;
9	(b) one or more target oligonucleotide duplexes deposited onto said
10	electrode or addressable multielectrode array, wherein each of said target
11	oligonucleotide duplexes is comprised of a first single-stranded nucleic acid
12	hybridized to a second single-stranded nucleic acid, and wherein one of said nucleic
13	acids contains a second single-stranded overhang complementary to said first single-
14	stranded overhang on said electrode or addressable multielectrode array; and
15	(c) an intercalative moiety site-specifically adsorbed to said target
16	oligonucleotide duplexes, wherein said intercalative moiety is daunomycin, and
17	wherein said target oligonucleotide duplexes provide electrical contact between the
18	electrode or addressable multielectrode array and daunomycin.

		50	
i	26.	A method of detecting one or more base-stacking perturbations in a	
2	target sequence comprising:		
3		(a) hybridizing a first single stranded nucleic acid to a second	
4	single strande	ed nucleic acid to form a first complex;	
5		(b) depositing said first complex onto an electrode or an	
6	addressable m	nultielectrode array;	
7		(c) adding an intercalative, redox-active moiety to said first	
8	complex to fo	orm a second complex; and	
9		(d) measuring an electron transfer event between said electrode or	
10	addressable multielectrode array and said intercalative, redox-active moiety as an		
11	indication for	the presence or absence of said base-stacking perturbations.	
1	27.	A method according to claim 26, wherein said base-stacking	
2	perturbations	are point mutations, protein-DNA adducts, adducts between any	
3	chemical enti	ty and said target sequence, or combinations thereof.	
1	28.	A method according to claim 26, wherein said intercalative, redox-	
2	active moiety	is either noncovalently adsorbed or crosslinked to said first complex.	
1	29.	A method according to claim 26, wherein said intercalative, redox-	
2	active moiety	is an intercalator.	
1	30.	A method according to claim 26, wherein said intercalative, redox-	
2	active moiety	is an intercalator selected from the group consisting of phenanthridines,	
3	phenothiazin	es, phenazines, acridines, and anthraquinones.	
1	31.	A method according to claim 26, wherein said intercalative, redox-	
2	active moiety	y is daunomycin.	
1	32.	A method according to claim 26, wherein said intercalative, redox-	
2	active moiety	y is part of a protein.	
1	33.	A method according to claim 26, wherein said intercalative, redox-	

2

active moiety is mut Y.

3

4 5

6

7

8

I

- 1 34. A method according to claim 26, wherein said electrode or addressable 2 multielectrode array is gold.
- 1 35. A method according to claim 26, wherein said electrode or addressable multielectrode array is carbon.
- 1 36. A method according to claim 26, wherein one of said single-stranded 2 nucleic acids is derivatized with a functionalized linker.
- 1 37. A method according to claim 36, wherein said functionalized linker is 2 comprised of 5 to 20 σ bonds.
- 1 38. A method according to claim 36, wherein said functionalized linker is 2 thiol-terminated.
- 1 39. A method according to claim 36, wherein said functionalized linker is 2 amine-terminated.
 - 40. A method according to claim 26, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a first single-stranded overhang of known sequence composition, and wherein one of said single-stranded nucleic acids contains a second single-stranded overhang complementary to said first single-stranded overhang on said electrode or addressable multielectrode array.
 - 41. A method of detecting one or more base-stacking perturbations in a target sequence comprising:
- (a) hybridizing a first single stranded nucleic acid to a second
 single stranded nucleic acid to form a first complex, wherein said nucleic acids are
 comprised of 12 to 25 nucleotides, and wherein one of said single-stranded nucleic
 acids is derivatized with a thiol-terminated linker comprised of 5 to 20 σ bonds;
- 7 (b) depositing said first complex onto an addressable gold 8 multielectrode array;

WO 99/51778 PCT/US99/07650

9	(c) adding daunomycin to said electrode-bound first complex to
10	form a second complex; and
11	(d) measuring an electron transfer event between said addressable
12	gold multielectrode array and daunomycin as an indication for the presence or absence
13	of said base-stacking perturbations.
1	42. A method according to claim 41, wherein said base-stacking
2	perturbations are point mutations, protein-DNA adducts, adducts between any
3	chemical entity and said target sequence, or combinations thereof.
1	43. A method of detecting one or more base-stacking perturbations in a
2	target sequence comprising:
3	(a) hybridizing a first single stranded nucleic acid to a second
4	single stranded nucleic acid to form a first complex, wherein said nucleic acids are
5	comprised of 12 to 25 nucleotides, and wherein one of said single-stranded nucleic
6	acids is derivatized with a amine-terminated linker comprised of 5 to 20 σ bonds;
7	(b) depositing said first complex onto an addressable carbon
8	multielectrode array;
9	(c) adding daunomycin to said electrode-bound first complex to
10	form a second complex; and
11	(d) measuring an electron transfer event between said addressable
12	carbon multielectrode array and daunomycin as an indication for the presence or
13	absence of said base-stacking perturbations.
1	44. A method according to claim 43, wherein said base-stacking
2	perturbations are point mutations, protein-DNA adducts, adducts between any
3	chemical entity and said target sequence, or combinations thereof.
1	45. A method of detecting one or more base-stacking perturbations in a
2	target sequence comprising:
3	(a) hybridizing a first single-stranded nucleic acid to a second
4	single-stranded nucleic acid to form a first complex of 12 to 25 nucleotides in length.

5	wherein said first complex contains a first single-stranded overhang of known		
6	sequence composition, and wherein said first single-stranded overhang can be the		
7	same or different;		
8	(b) depositing said first complex onto an addressable		
9	multielectrode array, wherein said addressable multielectrode array is comprised of a		
10	monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto		
11	said array, wherein each of said oligonucleotide duplexes is derivatized on one end		
12	with a functionalized linker and on the opposite end with a second single-stranded		
13	overhang complementary to said first single-stranded overhang;		
14	(c) adding daunomycin to said electrode-bound first complex to		
15	form a second complex; and		
16	(d) measuring an electron transfer event between said addressable		
17	multielectrode array and daunomycin as an indication for the presence or absence of		
18	said base-stacking perturbations.		
1	46. A method according to claim 45, wherein said base-stacking		
2	perturbations are point mutations, protein-DNA adducts, adducts between any		
3	chemical entity and said target sequence, or combinations thereof.		
1	47. A method of continuously detecting one or more base-stacking		
2	perturbations comprising:		
3	(a) hybridizing a single stranded nucleic acid derivatized with a		
4	functionalized linker to a first target sequence to form a first complex;		
5	(b) depositing said first complex onto an electrode or an		
6	addressable multielectrode array;		
7	(c) adsorbing an intercalative, redox-active moiety to said		
8	electrode-bound first complex to form a second complex;		
9	(d) measuring an electron transfer event between said electrode or		
10	addressable multielectrode array and said intercalative, redox-active moiety in said		

	31			
11	second complex as an indication for the presence or absence of said base-stacking			
12	perturbations;			
13	(e) disrupting said second complex by applying denaturing			
14	conditions, removing said first target sequence and said intercalative, redox-active			
15	noiety, and generating a single-stranded monolayer;			
16	(f) adding a second target sequence to said single-stranded			
17	nonolayer under hybridizing conditions to form a third complex;			
18	(g) adsorbing an intercalative, redox-active moiety to said third			
19	complex to form a fourth complex;			
20	(h) measuring an electron transfer event between said electrode or			
21	addressable multielectrode array and said intercalative, redox-active moiety in said			
22	fourth complex as an indication for the presence or absence of said base-stacking			
23	perturbations; and			
24	(i) repeating steps (e) through (h) using multiple target sequences.			
1	48. A method according to claim 47, wherein said base-stacking			
2	perturbations are point mutations, protein-DNA adducts, adducts between any			
3	chemical entity and said target sequence, or combinations thereof.			
1	49. A method according to claim 47, wherein said intercalative, redox-			
2	active moiety is an intercalator.			
1	50. A method according to claim 47, wherein said intercalative, redox-			
2	active moiety is an intercalator selected from the group consisting of phenanthridines.			
3	phenothiazines, phenazines, acridines, and anthraquinones.			
1	51. A method according to claim 47, wherein said intercalative, redox-			
2	active moiety is daunomycin.			
1	52. A method according to claim 47, wherein said intercalative, redox-			
2	active moiety is part of a protein.			
1	53. A method according to claim 47, wherein said intercalative, redox-			

active moiety is mut Y.

- 1 54. A method according to claim 47, wherein said electrode or addressable 2 multielectrode array is gold.
- 1 55. A method according to claim 47, wherein said electrode or addressable 2 multielectrode array is carbon.
- 1 56. A method according to claim 47, wherein said functionalized linker is comprised of 5 to 20 σ bonds.
- 1 57. A method according to claim 47, wherein said functionalized linker is 2 thiol-terminated.
- 1 58. A method according to claim 47, wherein said functionalized linker is 2 amine-terminated.
 - 59. A method of continuously detecting one or more base-stacking perturbations comprising:
- 3 (a) hybridizing a first single stranded nucleic acid to a second 4 single-stranded nucleic acid to form a first target duplex, wherein said first target 5 duplex contains a single-stranded overhang of known sequence composition, and 6 wherein said first single-stranded overhang can be the same or different;
- 7 (b) depositing said first target duplex onto an electrode or
 8 addressable multielectrode array to form a first complex, wherein said addressable
 9 multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to
 10 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide
 11 duplexes is derivatized on one end with a functionalized linker and on the opposite
 12 end with a second single-stranded overhang complementary to said first single13 stranded overhang;
- (c) adsorbing an intercalative, redox-active moiety to said first
 complex to form a second complex;
- (d) measuring an electron transfer event between said electrode or
 addressable multielectrode array and said intercalative, redox-active moiety in said

18	second complex as an indication for the presence or absence of said base-stacking		
19	perturbations;		
20 21 22	(e) disrupting said second complex by applying denaturing conditions and removing said first target duplex and said intercalative, redox-active moiety;		
23 24	(f) adding a second target duplex containing a single-stranded overhang under hybridizing conditions to form a third complex;		
25 26	(g) adsorbing an intercalative, redox-active moiety to said third complex to form a fourth complex;		
27 28 29 30	(h) measuring an electron transfer event between said electrode of addressable multielectrode array and said intercalative, redox-active moiety in said fourth complex as an indication for the presence or absence of said base-stacking perturbations; and		
31	(i) repeating steps (e) through (h) using multiple target duplexes.		
1 2 3	60. A method according to claim 59, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.		
1 2	61. A method according to claim 59, wherein said intercalative, redox-active moiety is an intercalator.		
1 2 3	62. A method according to claim 59, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines phenothiazines, phenazines, acridines, and anthraquinones.		
1 2	63. A method according to claim 59, wherein said intercalative, redox-active moiety is daunomycin.		
1 2	64. A method according to claim 59, wherein said intercalative, redoxactive moiety is part of a protein.		
1	65. A method according to claim 59, wherein said intercalative, redox-		

active moiety is mut Y.

1	66.	A met	hod of continuously detecting one or more base-stacking
2	perturbations comprising:		
3		(a)	hybridizing a single stranded nucleic acid which is derivatized
4	with a thiol-te	rminate	ed linker comprised of 5 to 20 o bonds to a first nucleic acid
5			m a first complex, wherein said nucleic acids are comprised of
6	12 to 25 nucle		
7		(b)	depositing said first complex onto an addressable gold
8	multielectrode	. ,	depositing same and
o	manneteerroad	-	
9		(c)	adsorbing daunomycin to said first complex to form a second
10	complex;		
11		(d)	measuring an electron transfer event between said gold
12	multielectrode	e array	and daunomycin in said second complex as an indication for the
13	presence or al	sence	of said base-stacking perturbations;
14		(e)	disrupting said second complex by immersing it in aqueous
15	solution at ele	evated t	emperatures for 1 minute, removing said first target sequence and
16			nerating a single-stranded monolayer;
17		(f)	adding a second target sequence to said single-stranded
18	monolayer ur	der hyl	oridizing conditions to form a third complex;
19		(g)	adsorbing daunomycin to said third complex to form a fourth
20	complex;	(6)	
21		(h)	measuring an electron transfer event between said gold
22	multielectrod	` '	and daunomycin in said fourth complex as an indication for the
23			of said base-stacking perturbations; and
24		(i)	repeating steps (e) through (h) using multiple target sequences.
l	67.	A me	ethod according to claim 66, wherein said base-stacking
2	perturbations	s are po	int mutations, protein-DNA adducts, adducts between any
3	chemical entity and said target sequence, or combinations thereof.		

1	68.	A me	thod of continuously detecting one or more base-stacking	
2	perturbations comprising:			
3		(a)	hybridizing a single stranded nucleic acid which is derivatized	
4	with an amin	e-termii	nated linker comprised of 5 to 20 σ bonds to a first nucleic acid	
5	target sequen	ce to fo	rm a first complex, wherein said nucleic acids are comprised of	
6	12 to 25 nucl	eotides		
7		(b)	depositing said first complex onto an addressable carbon	
8	multielectrod	e array;		
9		(c)	adsorbing daunomycin to said first complex to form a second	
10	complex;			
11		(d)	measuring an electron transfer event between said carbon	
12	multielectrod	e array	and daunomycin in said second complex as an indication for the	
13	presence or absence of said base-stacking perturbations;			
14		(e)	disrupting said second complex by immersing it in aqueous	
15	solution at elevated temperatures for 1 minute, removing said first target sequence and			
16	daunomycin.	and ger	nerating a single-stranded monolayer;	
17		(f)	adding a second target sequence to said single-stranded	
18	monolayer ui	nder hy	bridizing conditions to form a third complex:	
19		(g)	adsorbing daunomycin to said third complex to form a fourth	
20	complex;			
21		(h)	measuring an electron transfer event between said carbon	
22	multielectroc	le array	and daunomycin in said fourth complex as an indication for the	
23	presence or a	bsence	of said base-stacking perturbations; and	
24		(i)	repeating steps (e) through (h) using multiple target sequences.	
1	69.	A me	ethod according to claim 68, wherein said base-stacking	
2	perturbations	s are po	int mutations, protein-DNA adducts, adducts between any	
3	chemical entity and said target sequence, or combinations thereof.			

1	70. A method of continuously detecting one or more base-stacking
2	perturbations comprising:
3	(a) hybridizing a first single stranded nucleic acid to a second
4	single-stranded nucleic acid to form a first target duplex of 12 to 25 nucleotides in
5	length, wherein said first target duplex contains a single-stranded overhang of known
6	sequence composition, and wherein said first single-stranded overhang can be the
7	same or different;
8	(b) depositing said first target duplex onto an addressable
9	multielectrode array to form a first complex, wherein said addressable multielectrode
10	array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in
11	length deposited onto said array, wherein each of said oligonucleotide duplexes is
12	derivatized on one end with a functionalized linker and on the opposite end with a
13	second single-stranded overhang complementary to said first single-stranded
14	overhang;
15	(c) adsorbing daunomycin to said first complex to form a second
16	complex;
17	(d) measuring an electron transfer event between said addressable
18	multielectrode array and daunomycin in said second complex as an indication for the
19	presence or absence of said base-stacking perturbations;
20	(e) disrupting said second complex by immersing it in aqueous
21	solution at elevated temperatures for 1 minute, removing said first target duplex and
22	daunomycin, and regenerating said monolayer of oligonucleotide duplexes;
23	(f) adding a second target duplex to said monolayer of
24	oligonucleotide duplexes under hybridizing conditions, forming a third complex;
25	(g) adsorbing daunomycin to said third complex to form a fourth
26	complex;
27	(h) measuring an electron transfer event between said addressable
28	multielectrode array and daunomycin in said fourth complex as an indication for the
29	presence or absence of said base-stacking perturbations; and

30	(i) repeating steps (e) through (h) using multiple target duplexes.			
1	71. A method according to claim 70, wherein said base-stacking			
2	perturbations are point mutations, protein-DNA adducts, adducts between any			
3	chemical entity and said target sequence, or combinations thereof.			
1	72. A method of detecting one or more point mutations within the p53			
2	gene, comprising:			
3	(a) forming a set of oligonucleotide duplexes of approximately 20			
4	base-pairs in length corresponding to the approximately 600 base pair long region			
5	within exons 5 through 8 of the p53 gene, wherein said oligonucleotide duplexes are			
6	derivatized with a thiol-terminated linker comprised of 5 to 20 σ bonds;			
7	(b) depositing said oligonucleotide duplexes onto an addressable			
8	gold multielectrode array;			
9	(c) denaturing said oligonucleotide duplexes by immersing them in			
10	aqueous solution at elevated temperatures for 1 minute and removing the			
11	complementary strands to form a single-stranded monolayer;			
12	(d) exposing said single-stranded monolayer to a first sample			
13	comprising PCR-amplified and fragmented p53 gene DNA under hybridizing			
14	conditions to form a first complex;			
15	(e) rinsing said electrode-bound first complex to remove any			
16	unhybridized material;			
17	(f) adsorbing daunomycin to said first complex by immersing it in			
18	a dilute solution of daunomycin to form a second complex;			
19	(g) measuring an electron transfer event between said gold			
20	multielectrode array and daunomycin in said second complex as an indication for the			
21	presence or absence of said point mutations;			
22	(h) denaturing said second complex by immersing it in an aqueous			
23	solution at elevated temperatures for 1 minute, removing daunomycin and the			

24	complementar	y strano	ds from said first sample, and regenerating said single-stranded
25	monolayer;		
26		(i)	exposing said single-stranded monolayer to a second sample
27	containing PC	R-amp	lified and fragmented p53 gene DNA under hybridizing
28	conditions to f	orm a t	hird complex; and
29		(k)	repeating steps (e) through (h) using several sample solutions.
1	73.	A met	hod of detecting one or more point mutations within the p53
2	gene, comprisi	ing:	
3		(a)	generating a first sample set of oligonucleotide duplexes of
4	approximately	20 bas	e-pairs in length corresponding to the approximately 600 base
5	pair long region within exons 5 through 8 of the p53 gene, wherein said		
6	oligonucleotide duplexes contain PCR-amplified and fragmented p53 gene DNA, and		
7	wherein said oligonucleotide duplexes contain a first single-stranded overhang of		
8	known compo	sition;	
9		(b)	depositing said oligonucleotide duplexes onto an addressable
9 10	multielectrode	` ,	depositing said oligonucleotide duplexes onto an addressable to form a first complex, wherein said addressable multielectrode
		array (
10	array is compr	array (to form a first complex, wherein said addressable multielectrode
10 11	array is compr length deposit	e array (rised of	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in
10 11 12	array is compr length deposit derivatized on	e array trised of ed onto	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in said array, wherein each of said oligonucleotide duplexes is
10 11 12 13	array is compr length deposit derivatized on	e array trised of ed onto	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in said array, wherein each of said oligonucleotide duplexes is ad with a functionalized linker and on the opposite end with a
10 11 12 13 14	array is compr length deposit derivatized on second single-	e array trised of ed onto	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in said array, wherein each of said oligonucleotide duplexes is ad with a functionalized linker and on the opposite end with a
10 11 12 13 14 15	array is comprisently derivatized on second single-overhang;	e array trised of ed onto	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in a said array, wherein each of said oligonucleotide duplexes is add with a functionalized linker and on the opposite end with a ed overhang complementary to said first single-stranded
10 11 12 13 14 15	array is comprisently derivatized on second single-overhang;	e array trised of ed onto	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in a said array, wherein each of said oligonucleotide duplexes is add with a functionalized linker and on the opposite end with a ed overhang complementary to said first single-stranded adsorbing daunomycin to said first complex by immersing it in
10 11 12 13 14 15 16	array is comprehength deposited derivatized on second single-overhang;	e array trised of eed onto one en erstrande (c) on of d.	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in a said array, wherein each of said oligonucleotide duplexes is add with a functionalized linker and on the opposite end with a ed overhang complementary to said first single-stranded adsorbing daunomycin to said first complex by immersing it in aunomycin to form a second complex;
10 11 12 13 14 15 16 17	array is comprehength deposited derivatized on second single-overhang; a dilute solution multielectrode	e array frised of ed onto one en errande (c) on of de (d) e array	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in a said array, wherein each of said oligonucleotide duplexes is add with a functionalized linker and on the opposite end with a ed overhang complementary to said first single-stranded adsorbing daunomycin to said first complex by immersing it in aunomycin to form a second complex; measuring an electron transfer event between said
10 11 12 13 14 15 16 17 18	array is comprehength deposited derivatized on second single-overhang; a dilute solution multielectrode	e array frised of ed onto one en errande (c) on of de (d) e array	to form a first complex, wherein said addressable multielectrode a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in a said array, wherein each of said oligonucleotide duplexes is add with a functionalized linker and on the opposite end with a ed overhang complementary to said first single-stranded adsorbing daunomycin to said first complex by immersing it in aunomycin to form a second complex; measuring an electron transfer event between said and daunomycin in said second complex as an indication for the

23	oligonucleotid	e duple	xes, and regenerating said monolayer of oligonucleotide
24	duplexes;		
25		(f)	exposing said monolayer to a second sample set of
26	oligonucleotid	e duple	xes to form a third complex, wherein said oligonucleotide
27	duplexes conta	ain PCF	R-amplified and fragmented p53 gene DNA, and wherein said
28	oligonucleotid	e duple	xes contain a third single-stranded overhang complementary to
29	said second sin	ngle-str	anded overhang; and
30		(h)	repeating steps (c) through (f) using several sample sets.
1	74.	A met	hod of detecting one or more base-stacking perturbations
2	electrocatalyti	cally in	a target sequence comprising:
3		(a)	hybridizing a first single stranded nucleic acid to a second
4	single strande	d nucle	ic acid to form a first complex;
5		(c)	depositing said first complex onto an electrode or an
6	addressable m	ultielec	trode array to form a second complex;
7		(d)	immersing said second complex in a solution comprising an
8	intercalative,	redox-a	ctive species and a non-intercalative, redox-active species; and
9		(d)	measuring an electron transfer event as an indication for the
10	presence or ab	sence o	of said base-stacking perturbations.
1	75.	A met	hod according to claim 74, wherein said base-stacking
2	perturbations	are poi	nt mutations, protein-DNA adducts, adducts between any
3	chemical entit	ty and s	aid target sequence, or combinations thereof.
1	76.	A met	hod according to claim 74, wherein said intercalative, redox-
2	active moiety	is an ir	ntercalator.
1	77.	A me	thod according to claim 74, wherein said intercalative, redox-
2	active moiety	is an ir	ntercalator selected from the group consisting of phenanthridines,
3	phenothiazine	es, pher	nazines, acridines, and anthraquinones.

	03
1	78. A method according to claim 74, wherein said non-intercalative, redox-
2	active moiety is selected from the group consisting of ferricyanide, ferrocenes,
3	hexacyanoruthenate, and hexacyanoosmate.
1	79. A method according to claim 74, wherein said intercalative, redox-
2	active moiety is a protein.
1	80. A method according to claim 74, wherein said intercalative, redox-
2	active moiety is methylene blue, and wherein said non-intercalative, redox-active
3	moiety is ferricyanide.
1	81. A method according to claim 74, wherein said electrode or addressable
2	multielectrode array is gold.
1	82. A method according to claim 74, wherein said electrode or addressable
2	multielectrode array is carbon.
1	83. A method according to claim 74, wherein one of said single-stranded
2	nucleic acids is derivatized with a functionalized linker.
1	84. A method according to claim 83, wherein said functionalized linker is
2	comprised of 5 to 20 σ bonds.
1	85. A method according to claim 83, wherein said functionalized linker is
2	thiol-terminated.
1	86. A method according to claim 83, wherein said functionalized linker is
2	amine-terminated.
1	87. A method according to claim 74, wherein said addressable
2	multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to
3	10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide
4	duplexes are derivatized on one end with a functionalized linker and on the opposite
5	end with a first single-stranded overhang of distinct sequence composition, and

wherein one of said single-stranded nucleic acids contains a second single-stranded

overhang complementary to said first single-stranded overhang on said electrode or

6

7

8

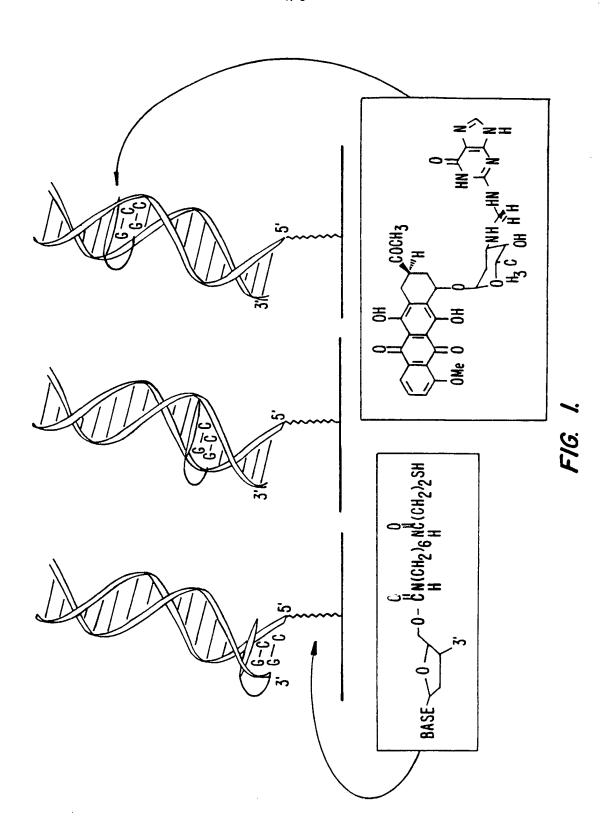
addressable multielectrode array.

1	88.	A met	hod of detecting one or more point mutations electrocatalytically
2	within the p53 gene, comprising:		
3		(a)	forming a set of oligonucleotide duplexes of approximately 20
4	base-pairs in l	ength c	orresponding to the approximately 600 base pair long region
5	within exons	5 throug	gh 8 of the p53 gene, wherein said oligonucleotide duplexes are
6	derivatized wi	ith a thi	ol-terminated linker comprised of 5 to 20 σ bonds;
7		(b)	depositing said oligonucleotide duplexes onto an addressable
8	gold multielectrode array;		
9		(c)	denaturing said oligonucleotide duplexes by immersing them in
10	aqueous solution at elevated temperatures for 1 minute and removing the		
11	complementary strands to form a single-stranded monolayer;		
12		(d)	exposing said single-stranded monolayer to a first sample
13	comprising PO	CR-amp	olified and fragmented p53 gene DNA under hybridizing
14	conditions to form a first complex;		
15		(e)	rinsing said electrode-bound first complex to remove any
16	unhybridized	materia	l;
17		(f)	immersing said electrode-bound first complex into a dilute
18	solution comp	rised of	f 1.0 μM methylene blue and 1.0 mM ferricyanide;
19		(g)	measuring an electron transfer event as an indication for the
20	presence or ab	sence o	of said point mutations;
21		(h)	denaturing said electrode-bound first complex by immersing it
22	in an aqueous	solutio	n at elevated temperatures for 1 minute, and regenerating said
23	single-strande	d mono	layer;
24		(i)	exposing said single-stranded monolayer to a second sample
25	containing PCR-amplified and fragmented p53 gene DNA under hybridizing		
26	conditions to form a second complex; and		
27		(k)	repeating steps (e) through (h) using several sample solutions.

1	89. A method of	of detecting one or more point mutations electrocatalytically
2	within the p53 gene, comp	orising:
3	(a) gen	erating a first sample set of oligonucleotide duplexes of
4	approximately 20 base-pai	rs in length corresponding to the approximately 600 base
5	pair long region within ex	ons 5 through 8 of the p53 gene, wherein said
6	oligonucleotide duplexes	contain PCR-amplified and fragmented p53 gene DNA, and
7	wherein said oligonucleoti	de duplexes contain a first single-stranded overhang of
8	known composition;	
9	(b) dep	ositing said oligonucleotide duplexes onto an addressable
0	gold multielectrode array	to form a first complex, wherein said addressable gold
11	multielectrode array is cor	nprised of a monolayer of oligonucleotide duplexes of 5 to
12	10 base-pairs in length dep	posited onto said array, wherein each of said oligonucleotide
13	duplexes is derivatized on	one end with a functionalized linker and on the opposite
14	end with a second single-s	stranded overhang complementary to said first single-
15	stranded overhang;	
16	(c) imr	nersing said electrode-bound first complex into a dilute
17	solution comprised of 1.0	μM methylene blue and 1.0 mM ferricyanide;
18	(d) mea	asuring an electron transfer event as an indication for the
19	presence or absence of sai	d point mutations;
20	(e) disa	rupting said second complex by immersing it in aqueous
21	solution at elevated temper	eratures for 1 minute, removing said first sample set of
22	oligonucleotide duplexes.	and regenerating said monolayer of oligonucleotide
23	duplexes;	
24	(f) exp	posing said monolayer to a second sample set of
25	oligonucleotide duplexes	to form a third complex, wherein said oligonucleotide
26	duplexes contain PCR-an	plified and fragmented p53 gene DNA, and wherein said
27	oligonucleotide duplexes	contain a third single-stranded overhang complementary to
28	said second single-strand	ed overhang; and
29	(h) rep	beating steps (c) through (f) using several sample sets.

I	90.	A me	thod of detecting the presence of a protein in a solution	
2	comprising:			
3		(a)	hybridizing a first single stranded nucleic acid to a second	
4	single strande	ed nucle	eic acid to form a first complex, wherein the sequence	
5	composition	of said	first complex comprises the recognition sequence of said protein;	
6		(b)	depositing said first complex onto an electrode or an	
7	addressable n	nultiele	ctrode array;	
8		(c)	adding an intercalative, redox-active moiety to said first	
9	complex to fo	orm a se	econd complex;	
10		(d)	immersing said second complex into a first sample solution	
11	which potentially comprises said protein;			
12		(e)	measuring an electron transfer event between said electrode or	
13	addressable n	nultiele	ctrode array and said intercalative, redox-active moiety of said	
14	second comp	d complex as an indication for the presence or absence of said protein in said		
15	first sample s	olution	;	
16		(f)	rinsing said second complex;	
17		(g)	immersing said second complex into a second sample solution	
18	which potent	ially co	mprises said protein; and	
19		(h)	repeating steps (e) through (g) using multiple sample solutions	
1	91.	A me	thod according to claim 90, wherein said intercalative, redox-	
2	active moiety	is eith	er noncovalently adsorbed or crosslinked to said first complex.	
1	92.	A me	thod according to claim 90, wherein said intercalative, redox-	
2	active moiety	is an i	ntercalator.	
1	93.	A me	ethod according to claim 90, wherein said intercalative, redox-	
2	active moiety	is an i	ntercalator selected from the group consisting of phenanthridines	
3	phenothiazin	es, phe	nazines, acridines, and anthraquinones.	
ì	94.	A me	ethod according to claim 90, wherein said intercalative, redox-	
2	active moiety	y is dau	nomycin.	

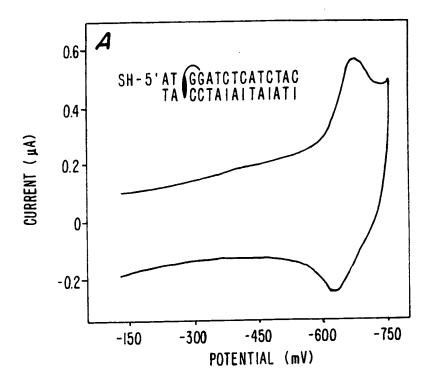
- 1 95. A method according to claim 90, wherein said electrode or addressable 2 multielectrode array is gold.
- 1 96. A method according to claim 90, wherein said electrode or addressable 2 multielectrode array is carbon.
- 1 97. A method according to claim 90, wherein one of said nucleic acids is 2 derivatized with a functionalized linker.
- 98. A method according to claim 97, wherein said functionalized linker is
 comprised of 5 to 20 σ bonds.
- 1 99. A method according to claim 97, wherein said functionalized linker is thiol-terminated.
- 1 100. A method according to claim 97, wherein said functionalized linker is 2 amine-terminated.
- 1 101. A method according to claim 90, wherein said addressable
 2 multielectrode array is comprised of oligonucleotide duplexes of 5 to 10 base-pairs in
 3 length deposited onto said array, wherein each of said oligonucleotide duplexes is
 4 derivatized on one end with a functionalized linker and on the opposite end with a
- 5 first single-stranded overhang of distinct sequence composition, and wherein one of
- 6 said nucleic acids has a second single-stranded overhang that is complementary to
- 7 said first single-stranded overhang on said addressable multielectrode array.



SUBSTITUTE SHEET (RULE 26)

WO 99/51778

2/9



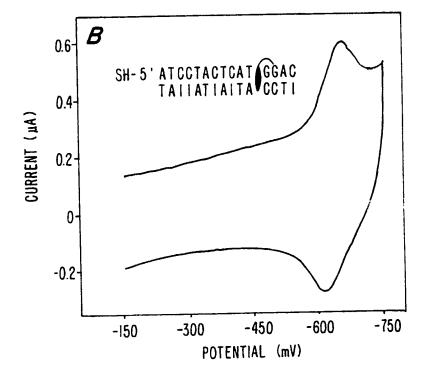


FIG. 2. SUBSTITUTE SHEET (RULE 26)

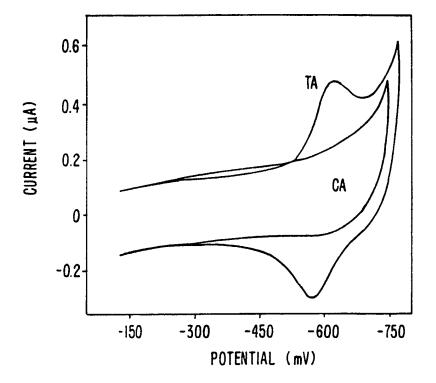


FIG. 3.

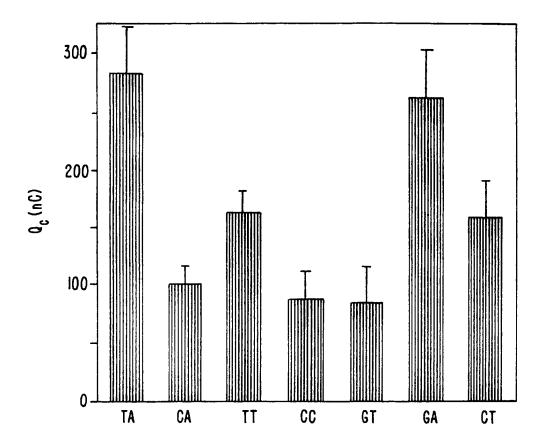
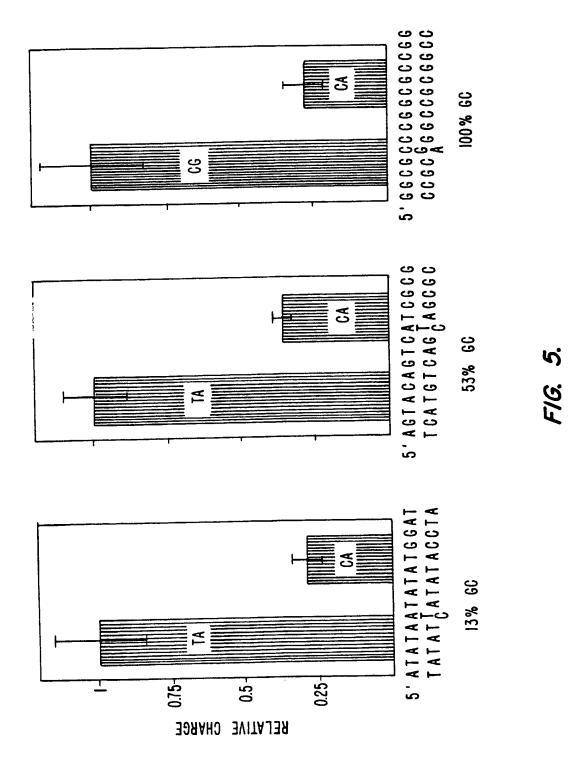
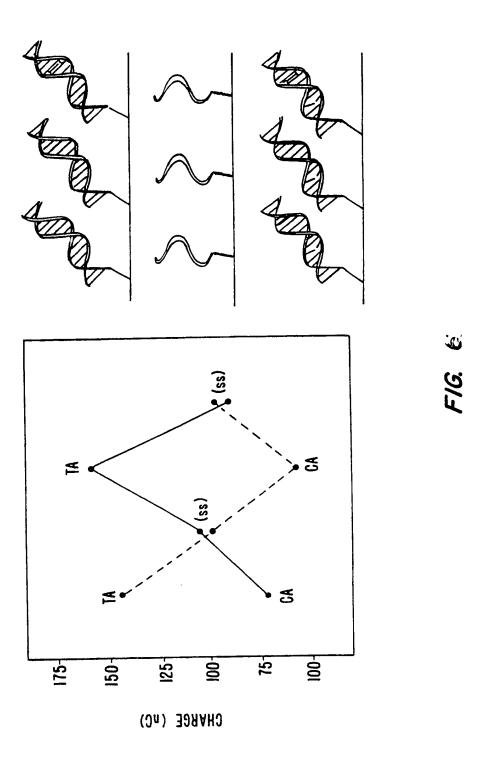


FIG. 4.



SUBSTITUTE SHEET (RULE 26)



SUBSTITUTE SHEET (RULE 26)

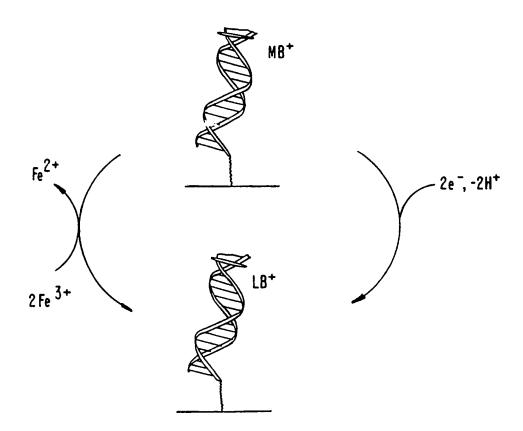


FIG. 7.

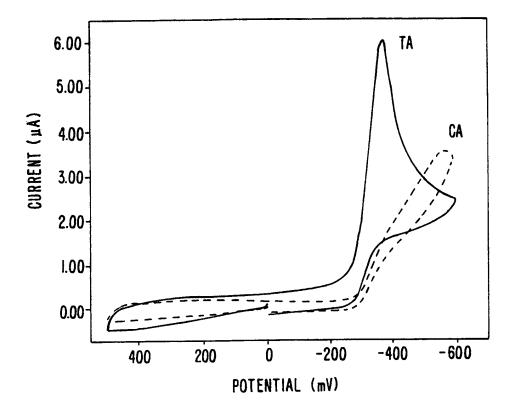


FIG. 8.

WO 99/51778

9/9

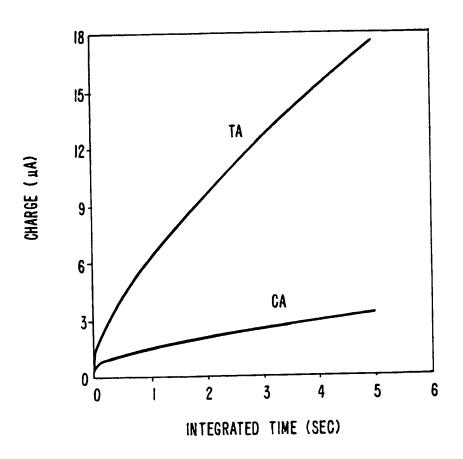


FIG. 9.

INTERNATIONAL SEARCH REPORT

Inte onal Application No PCT/US 99/07650

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12Q1/68 G011 G01N27/327 G01N33/50 According to International Patent Classification (IPC) or to both national classification and IPC B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C120 G01N IPC 6 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practical, search terms used) C. DOCUMENTS CONSIDERED TO BE RELEVANT Relevant to claim No. Citation of document, with indication, where appropriate, of the relevant passages 1,3,26, WO 95 15971 A (CALIFORNIA INSTITUTE OF Α TECHNOLOGY) 15 June 1995 (1995-06-15) 28,41, 43,45, 47,59, 66,68, 70,74 abstract; claims US 5 312 527 A (MIKKELSEN ET AL.) Α 22-26, 17 May 1994 (1994-05-17) 41,43, 45,47, 59,66, 68,70,74 column 4 - column 6; figure 3 -/--Patent family members are listed in annex. Further documents are listed in the continuation of box C. * Special categories of cited documents : "" later document published after the international filing date or priority date and not in conflict with the application but *A* document defining the general state of the art which is not considered to be of particular relevance cited to understand the principle or theory underlying the invention "E" earlier document but published on or after the international "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docucitation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or ments, such combination being obvious to a person skilled in the art. other means *P* document published prior to the international filing date but later than the priority date claimed "&" document member of the same patent family Date of mailing of the international search report Date of the actual completion of the international search 03 09 1999 7 July 1999 Authorized officer Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Mosser Fax: (+31-70) 340-3016

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 99/07650

HASHIMOTO, K. ET AL.: "Novel DNA sensor for electro chemical gene detection" ANALYTICA CHIMICA ACTA, vol. 286, 1994, pages 219-224, XP002900561 the whole document JOHNSTON, D.H. ET AL.: "Electrochemical heasurement of the Solvent Accessibility for Nucleobases Using Electron Transfer 28,74, between DNA and Metal Complexes" 78,90,91 J. CHEM. SOC., vol. 117, 1995, pages 8933-8938, XP002900562 page 8933; figures 1,2; tables 1,2 CHEMICAL ABSTRACTS, vol. 120, no. 11, 14 March 1994 (1994-03-14) 9-12,17, Columbus, Ohio, US; abstract no. 126263c, HASHIMOTO ET AL.: "DNA sensor: a novel electrochemical gene defection method using carbon electrode immobilized DNA probes" page 246; column 2; XP002900563 62,66, 74,76, 77,82 abstract & SUPRAMOL. CHEM., vol. 2, no. 4, 1993, pages 265-270,	Category °	ation) DOCUMENTS CONSIDERED TO BE RELEVANT Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
for electro chemical gene detection" ANALYTICA CHIMICA ACTA, vol. 286, 1994, pages 219-224, XP002900561 the whole document JOHNSTON, D.H. ET AL.: "Electrochemical 1,3,9, Measurement of the Solvent Accessibility 10,26, of Nucleobases Using Electron Transfer 28,74, between DNA and Metal Complexes" 28,74, between DNA and Metal Complexes" 78,90,91 J. CHEM. SOC vol. 117, 1995, pages 8933-8938, XP002900562 page 8933; figures 1,2; tables 1,2 CHEMICAL ABSTRACTS, vol. 120, no. 11, 14 March 1994 (1994-03-14) 9-12,17, Columbus, Ohio, US; abstract no. 126263c, HASHIMOTO ET AL.: "DNA sensor: a novel electrochemical gene defection method 43,45, using carbon electrode immobilized DNA probes" page 246; column 2; XP002900563 abstract & SUPRAMOL. CHEM., vol. 2, no. 4, 1993, pages 265-270, CHEMICAL ABSTRACTS, vol. 127, no. 2, 14 July 1997 (1997-07-14) Columbus, Ohio, US; abstract no. 13828s, MANG ET AL.: "Detection of point mutation in the p53 gene using a peptide nucleic acid biosensor" page 135; column 1; XP002900564 abstract & ANAL. CHIM. ACTA,	-eredory	Citation of occument, with indication, where appropriate, of the research passages	Transacto Galiti 140.
Measurement of the Solvent Accessibility of Nucleobases Using Electron Transfer between DNA and Metal Complexes" J. CHEM. SOC., vol. 117, 1995, pages 8933-8938, XP002900562 page 8933; figures 1,2; tables 1,2 A CHEMICAL ABSTRACTS, vol. 120, no. 11, 14 March 1994 (1994-03-14) Columbus, Ohio, US; abstract no. 126263c, HASHIMOTO ET AL.: "DNA sensor: a novel electrochemical gene defection method using carbon electrode immobilized DNA probes" page 246; column 2; XP002900563 abstract & SUPRAMOL. CHEM., vol. 2, no. 4, 1993, pages 265-270, CHEMICAL ABSTRACTS, vol. 127, no. 2, 14 July 1997 (1997-07-14) Columbus, Ohio, US; abstract no. 13828s, WANG ET AL.: "Detection of point mutation in the p53 gene using a peptide nucleic acid biosensor" page 135; column 1; XP002900564 abstract & ANAL. CHIM. ACTA,	A	for electro chemical gene detection" ANALYTICA CHIMICA ACTA, vol. 286, 1994, pages 219-224, XP002900561	
14 March 1994 (1994-03-14) Columbus, Ohio, US; abstract no. 126263c, HASHIMOTO ET AL.: "DNA sensor: a novel electrochemical gene defection method using carbon electrode immobilized DNA probes" page 246; column 2; XP002900563 A CHEMICAL ABSTRACTS, vol. 127, no. 2, 14 July 1997 (1997-07-14) Columbus, Ohio, US; abstract no. 13828s, WANG ET AL.: "Detection of point mutation in the p53 gene using a peptide nucleic acid biosensor" page 135; column 1; XP002900564 abstract & ANAL. CHIM. ACTA,	A	Measurement of the Solvent Accessibility of Nucleobases Using Electron Transfer between DNA and Metal Complexes" J. CHEM. SOC., vol. 117, 1995, pages 8933-8938, XP002900562	10,26,
vol. 2, no. 4, 1993, pages 265-270, CHEMICAL ABSTRACTS, vol. 127, no. 2, 14 July 1997 (1997-07-14) Columbus, Ohio, US; abstract no. 13828s, WANG ET AL.: "Detection of point mutation in the p53 gene using a peptide nucleic acid biosensor" page 135; column 1; XP002900564 abstract & ANAL. CHIM. ACTA,	A	14 March 1994 (1994-03-14) Columbus, Ohio, US; abstract no. 126263c, HASHIMOTO ET AL.: "DNA sensor: a novel electrochemical gene defection method using carbon electrode immobilized DNA probes" page 246; column 2; XP002900563	9-12,17, 24,26, 28-30, 35,41, 43,45, 47,49, 50,55, 59,61, 62,66, 74,76,
Columbus, Ohio, US; abstract no. 13828s, WANG ET AL.: "Detection of point mutation in the p53 gene using a peptide nucleic acid biosensor" page 135; column 1; XP002900564 abstract & ANAL. CHIM. ACTA,	A	vol. 2, no. 4, 1993, pages 265-270, CHEMICAL ABSTRACTS, vol. 127, no. 2,	
		Columbus, Ohio, US; abstract no. 13828s, WANG ET AL.: "Detection of point mutation in the p53 gene using a peptide nucleic acid biosensor" page 135; column 1; XP002900564 abstract & ANAL. CHIM. ACTA,	

INTERNATIONAL SEARCH REPORT

mormation on patent family members

Inter: Inal Application No PCT/US 99/07650

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9515971	A 15-06-1995	US 5591578 A AU 703329 B AU 1215295 A CA 2178618 A EP 0733058 A JP 9506510 T US 5824473 A US 5770369 A US 5780234 A US 5705348 A	07-01-1997 25-03-1999 27-06-1995 15-06-1995 25-09-1996 30-06-1997 20-10-1998 23-06-1998 14-07-1998 06-01-1998
US 5312527	A 17-05-1994	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)

PCT

WELTORGANISATION FÜR GEISTIGES EIGENTUM Internationales Büro



INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 7:

C07H 21/00, C12Q 1/68

(11) Internationale Veröffentlichungsnummer:

WO 00/31101

A1 (4

(43) Internationales Veröffentlichungsdatum:

2. Juni 2000 (02.06.00)

(21) Internationales Aktenzeichen:

PCT/EP99/08888

(22) Internationales Anmeldedatum:

19. November 1999

(19.11.99)

(30) Prioritätsdaten:

198 53 957.6 199 21 940.0 23. November 1998 (23.11.98) DE

29. April 1999 (29.04.99) DE

(71)(72) Anmelder und Erfinder: HARTWICH, Gerhard [DE/DE]; Nibelungenstrasse 10, D-80639 München (DE).

(72) Erfinder; und

(75) Erfinder/Anmelder (nur für US): HELLER, Adam [US/US]; 7531 Velbum Circle, Austin, TX 78731 (US).

(81) Bestimmungsstaaten: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA. CH, CN, CU, CZ, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RÜ, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht

Mit internationalem Recherchenbericht.

(54) Title: METHOD FOR THE ELECTROCHEMICAL DETECTION OF NUCLEIC ACID OLIGOMER HYBRIDS

(54) Bezeichnung: VERFAHREN ZUR ELEKTROCHEMISCHEN DETEKTION VON NUKLEINSÄUREOLIGOMERHYBRIDEN

(57) Abstract

The invention relates to a method for the electrochemical detection of sequence-specific nucleic acid oligomer hybridization events. To this end single DNA/RNA/PNA oligomer strands which at one end are covalently joined to a support surface and at the other, free end, covalently linked to a redox pair, are used as hybridization matrix (probe). As a result of treatment with the olignucleotide solution (target) to be examined, the electric communication between the conductive support surface and the redox pair bridged by the single-strand oligonucleotide, which communication initially is either absent or very weak, is modified. In case of hybridization, electric communication between the surface support and the redox pair, which is now bridged by a hybridized double-strand oligonucleotide, is increased. This permits the detection of a hybridization event by electrochemical methods such as cyclic voltametry, amperometry or conductivity measurement.

(57) Zusammenfassung

, · ; ;

Die vorliegende Erfindung betrifft ein Verfahren zur elektrochemischen Detektion von sequenzspezifischen Nukleinsäure-Oligomer-Hybridisierungsereignissen. Dabei di-

Trager- a operflacete

a...SUPPORT SURFACE

enen DNA-/RNA-/PNA-Oligomer-Einzelstränge, die mit einem Ende kovalent auf einer Trägeroberfläche angebunden und am anderen, freien Ende kovalent mit einem Redoxpaar verknüpft sind, als Hybridisierungsmatrix ("Sonde"). Durch Behandlung mit der zu untersuchenden Oligonukleotid-Lösung ("Target") wird die ursprünglich nicht oder nur schwach vorhandene elektrische Kommunikation zwischen der leitfähigen Trägeroberfläche und dem über Einzelstrang-Oligonukleotid verbrückten Redoxpaar verändert. Im Falle der Hybridisierung wird die elektrische Kommunikation zwischen der Trägeroberfläche und dem nunmehr über hybridisiertes Doppelstrang-Oligonukleotid verbrückten Redoxpaar verstärkt. Somit wird die Detektion eines Hybridisierungsereignisses durch elektrochemische Verfahren wie cyclische Voltametrie, Amperometrie oder Leitfähigkeitsmessung ermöglicht.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AL	Albanien	ES	Spanien	LS	Lesotho	SI	Slowenien
AM	Armenien	FI	Finnland	LT	Litauen	SK	Slowakei
AT	Österreich	FR	Frankreich	LU	Luxemburg	SN	Senegal
AU	Australien	GA	Gabun	LV	Lettland	SZ.	Swasiland
AZ	Aserbaidschan	GB	Vereinigtes Königreich	MC	Monaco	TD	Tschad
BA	Bosnien-Herzegowina	GE	Georgien	MD	Republik Moldau	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagaskar	ТJ	Tadschikistan
BE	Belgien	GN	Guinea	MK	Die ehemalige jugoslawische	TM	Turkmenistan
BF	Burkina Faso	GR	Griechenland		Republik Mazedonien	TR	Türkei
BG	Bulgarien	HU	Ungam	ML	Mali	TT	Trinidad und Tobago
BJ	Benin	Œ	Irland	MN	Mongolei	UA	Ukraine
BR	Brasilien	IL	Israel	MR	Mauretanien	UG	Uganda
BY	Belarus	IS	Island	MW	Malawi	US	Vereinigte Staaten vo
CA	Kanada	IT	Italien	MX	Mexiko		Amerika
CF	Zentralafrikanische Republik	JР	Japan	NE	Niger	UZ	Usbekistan
CG	Kongo	KE	Kenia	NL	Niederlande	VN	Vietnam
CH	Schweiz	KG	Kirgisistan	NO	Norwegen	YU	Jugoslawien
CI	Côte d'Ivoire	KP	Demokratische Volksrepublik	NZ	Neuseeland	ZW	Zimbabwe
CM	Kamerun		Korea	PL	Polen		
CN	China	KR	Republik Korea	PT	Portugal		
CU	Kuba	ΚZ	Kasachstan	RO	Rumänien		
CZ	Tschechische Republik	LC	St. Lucia	RU	Russische Föderation		
DE	Deutschland .	LI	Liechtenstein	SD	Sudan		
DK	Dänemark	LK	Sri Lanka	SE	Schweden		
EE	Estland	LR	Liberia	SG	Singapur		

WO 00/31101 PCT/EP99/08888

Verfahren zur elektrochemischen Detektion von Nukleinsäureoligomerhybriden

Technisches Gebiet

Die vorliegende Erfindung betrifft ein modifiziertes Nukleinsäure-Oligomer, sowie ein Verfahren zur elektrochemischen Detektion von sequenzspezifischen Nukleinsäure-Oligomer-Hybridisierungsereignissen.

Stand der Technik

Zur Sequenzanalyse von DNA und RNA, z. B. in der Krankheitsdiagnose, bei toxikologischen Testverfahren, in der genetischen Forschung und Entwicklung, sowie auf dem Agrar- und pharmazeutischen Sektor, werden im allgemeinen gelelektrophoresische Verfahren mit autoradiographischer oder optischer Detektion verwendet.

Zur Veranschaulichung des wichtigsten gel-elektrophoretischen Verfahrens mit optischer Detektion (Sanger-Verfahren) ist in Figur 1b ein DNA-Fragment mit Primer dargestellt. Bei dem Sanger-Verfahren wird eine DNA enthaltende Lösung in vier Ansätze aufgeteilt und der Primer jedes Ansatzes mit je einem bei verschiedener Wellenlänge emitierenden Fluoreszenzfarbstoff kovalent modifiziert. Wie in Figur 1b dargestellt wird zu jedem Ansatz Desoxyribonucleosid-Triphosphat der Basen A (Adenin), T (Thymin), C (Cytosin), und G (Guanin), also dATP, dTTP, dCTP und dGTP, gegeben, um den Einzelstrang, ausgehend vom Primer, durch DNAenzymatisch zu replizieren. l Zusätzlich zu Desoxyribonucleosid-Triphosphaten enthält jedes Reaktionsgemisch noch genügend des 2',3'-Didesoxyanalogons (Figur 1a) eines dieser Nukleosidtriphosphate als Stopbase (je eine der 4 möglichen Stoppbasen pro Ansatz), um die Replikation an allen möglichen Bindungsstellen zu stoppen. Nach Vereinigung der vier Ansätze entstehen replizierte DNA-Fragment aller Längen mit stopbasenspezifischer Fluoreszenz, die gel-elektrophoretisch der Länge nach sortiert und durch Fluoreszenz-Spektroskopie charakterisiert werden können (Figur 1c).

Ein anderes optisches Detektionsverfahren basiert auf der Anlagerung von Fluoreszenzfarbstoffen wie z. B. Ethidiumbromid an Oligonukleotide. Die Fluoreszenz solcher Farbstoffe steigt im Vergleich zur freien Lösung des Farbstoffs um etwa das 20-fache an, wenn sie sich an doppelsträngige DNA oder RNA

anlagern und kann deshalb zum Nachweis hybridisierter DNA oder RNA verwendet werden.

Bei der radioaktiven Markierung wird ³²P in das Phosphatgerüst der Oligonukleotide eingebaut, wobei ³²P gewöhnlich am 5'-Hydroxylende durch Polynukleotid-Kinase addiert wird. Die markierte DNA wird anschließend an jeweils einem der vier Nukleotidtypen bevorzugt gespalten und zwar unter definierten Bedingungen, so daß pro Kette durchschnittlich eine Spaltung erfolgt. Damit liegen im Reaktionsgemisch für einen bestimmten Basentyp Ketten vor, die sich von der ³²P-Markierung bis zur Position dieser Base erstrecken (bei mehrfachem Auftreten der Base erhält man entsprechend Ketten unterschiedlicher Länge). Die vier Fragmentgemische werden anschließend auf vier Bahnen gel-elektrophoretisch aufgetrennt. Danach wird vom Gel ein Autoradiogramm angefertigt, an dem die Sequenz unmittelbar abgelesen werden kann.

Vor einigen Jahren wurde ein weiteres, auf optischer (oder autoradiographischer) Detektion beruhendes Verfahren zur DNA-Sequenzierung entwickelt, nämlich die Sequenzierung durch Oligomerhybridisierung (vgl. z. B. Drmanac et al., Genomics 4, (1989), S. 114-128 oder Bains et al., Theor. Biol. 135, (1988), S. 303-307). Bei diesem Verfahren wird ein vollständiger Satz kurzer Oligonukleotide bzw. Oligomere (Sonden-Oligonukleotide), z. B. alle 65 536 möglichen Kombinationen der Basen A, T, C und G eines Oligonukleotid-Oktamers auf ein Trägermaterial gebunden. Die Anbindung geschieht in einem geordneten Raster aus 65 536 Test-Sites, wobei jeweils eine größere Menge einer Oligonukleotid-Kombination ein Test-Site definieren und die Position jeder einzelnen Test-Site (Oligonukleotid-Kombination) bekannt ist. Auf solch einer Hybridisierungsmatrix, dem Oligomerchip, wird ein DNA-Fragment, dessen Sequenz man ermitteln will, das Target, mit Fluoreszenz-Farbstoff ³²P) markiert und unter Bedingungen, die nur eine spezifische Doppelstrangbildung erlauben, hybridisiert. Dadurch bindet das Target DNA-Fragment nur an die Oligomere (im Beispiel an die Oktamere), deren komplementäre Sequenz exakt einem Teil (einem Oktamer) seiner eigenen Sequenz entspricht. Durch optische (oder autoradiographische) Detektion der Bindungsposition des hybridisierten DNA-Fragments werden damit alle im Fragment vorhandenen Oligomerseguenzen (Oktamerseguenzen) bestimmt. Aufgrund der Überlappung Oligomersequenzen kann durch geeignete mathematische benachbarter Algorithmen die fortlaufende Sequenz des DNA-Fragments bestimmt werden. Die Vorteile dieses Verfahrens liegen unter anderem in der Miniaturisierung der Sequenzierung und damit in der enormen Datenmenge, die gleichzeitig in einem Arbeitsgang erfaßt wird. Daneben kann auf Primer und auf das gelelektrophoretische Auftrennen der DNA-Fragmente verzichtet werden. Beispielhaft ist dieses Prinzip in Figur 2 für ein 13 Basen langes DNA-Fragment gezeigt.

Die Verwendung radioaktiver Markierungen bei der DNA-/RNA- Sequenzierung ist mit mehreren Nachteilen verbunden, wie z. B. aufwendige, gesetzlich vorgeschriebene Sicherheitsvorkehrungen beim Umgang mit radioaktiven Materialien, die Strahlenbelastung, das begrenzte räumliche Auflösungsvermögen (maximal 1mm²) und eine Sensitivität, die nur dann hoch ist, wenn die Strahlung der radioaktiven Fragmente entsprechend lange (Stunden bis Tage) auf einen Röntgenfilm einwirkt. Es kann zwar die räumliche Auflösung durch zusätzliche Hardund Software erhöht und die Detektionszeit durch die Verwendung von β -Scannern verkürzt werden, beides ist jedoch mit erheblichen zusätzlichen Kosten verbunden.

Die Fluoreszenzfarbstoffe, die üblicherweise zur Markierung der DNA verwendet werden, sind zum Teil (z. B. Ethidiumbromid) mutagen und erfordern, ebenso wie die Anwendung der Autoradiographie, entsprechende Sicherheitsvorkehrungen. In fast allen Fällen erfordert die Verwendung optischer Detektion den Gebrauch von einem oder mehreren Lasersystemen und somit geschultes Personal und entsprechende Sicherheitsvorkehrungen. Die eigentliche Detektion der Fluoreszenz erfordert zusätzliche Hardware, wie z. B. optische Bauelemente zur Verstärkung und, bei verschiedenen Anregungs- und Abfragewellenlängen wie im Sanger-Verfahren, ein Kontrollsystem. Abhängig von den benötigten Anregungswellenlängen und der gewünschten Detektionsleistung können somit erhebliche Investitionskosten entstehen. Bei der Sequenzierung durch Hybridisierung auf dem Oligomerchip ist die Detektion noch (kosten)aufwendiger, da, neben dem Anregungssystem, zur 2-dimensionalen Detektion der Fluoreszenzspots hochauflösende CCD-Kameras (Charge Coupled Device Kameras) benötigt werden.

Obwohl es also quantitative und extrem sensitive Methoden zur DNA-/RNA-Sequenzierung gibt, sind diese Methoden zeitaufwendig, bedingen aufwendige Probenpräparation und teure Ausstattung und sind im allgemeinen nicht als transportable Systeme verfügbar.

Darstellung der Erfindung

Aufgabe der vorliegenden Erfindung ist es deshalb, eine Vorrichtung und ein Verfahren zur Detektion von Nukleinsäureoligomerhybriden zu schaffen, welche die Nachteile des Standes der Technik nicht aufweisen.

Diese Aufgabe wird erfindungsgemäß durch das modifizierte Oligonukleotid gemäß unabhängigem Patentanspruch 1, durch das Verfahren zur Herstellung eines modifizierten Oligonukleotids gemäß unabhängigem Anspruch 9 und 10, durch die modifizierte leitfähige Oberfläche gemäß unabhängigem Patentanspruch 11, das Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche gemäß unabhängigem Patentanspruch 21, sowie durch das Verfahren zur elektrochemischen Detektion von Oligomerhybridisierungsereignissen gemäß unabhängigem Patentanspruch 27 gelöst.

Im Rahmen der vorliegenden Erfindung werden die folgenden Abkürzungen und Begriffe benutzt:

Genetik						
DNA	Desoxyribonukleinsäure					
RNA	Ribonukleinsäure					
PNA	Peptidnukleinsäure (synthetische DNA oder RNA, bei der die Zucker-Phosphat Einheit durch eine Aminosäure ersetzt ist. Bei Ersatz der Zucker-Phosphat Einheit durch die -NH-(CH ₂) ₂ -N(COCH ₂ -Base)-CH ₂ CO- Einheit hybridisiert PNA mit DNA).					
Α	Adenin					
G	Guanin					
С	Cytosin					
Т	Thymin					
Base	A, G, T, oder C					
Вр	Basenpaar					
Nukleinsäure	 wenigstens zwei kovalent verbundene Nukleotide oder					

kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- (z. B. Cytosin, Thymin oder Uracil) oder Purin-Basen (z. B. Adenin oder Guanin). Der Begriff Nukleinsäure bezieht sich auf ein beliebiges "Rückgrat" der kovalent verbundenen Pyrimidin- oder Purin-Basen, wie z. B. auf das Zucker-Phosphat Rückgrat der DNA, cDNA oder RNA, auf ein Peptid-Rückgrat der PNA oder auf analoge Strukturen (z. B. Phosphoramid-, Thio-Phosphatoder Dithio-Phosphat-Rückgrat). Wesentliches Merkmal einer

Nukleinsäure im Sinne der vorliegenden Erfindung ist, daß sie natürlich vorkommende cDNA oder RNA sequenzspezifisch binden kann.

Nukleinsäure-Oligomer Nukleinsäure nicht näher spezifizierter Basenlänge (z. B. Nukleinsäure-Oktamer: eine Nukleinsäure mit beliebigem Rückgrat, bei dem 8 Pyrimidin- oder Purin-Basen kovalent aneinander gebunden sind).

Oligomer

Äquivalent zu Nukleinsäure-Oligomer.

Oligonukleotid

Äquivalent zu Oligomer oder Nukleinsäure-Oligomer, also z. B. ein DNA, PNA oder RNA Fragment nicht näher spezifizierter Basenlänge.

Oligo

Abkürzung für Oligonukleotid.

dATP

Desoxyribonucleosid-Triphosphat des A (DNA-Einheit mit der Base A und zwei weiteren Phosphaten zum Aufbau eines längeren DNA-Fragments bzw. eines Oligonukleotids).

dGTP

Desoxyribonucleosid-Triphosphat des G (DNA-Einheit mit der Base G und zwei weiteren Phosphaten zum Aufbau eines längeren DNA-Fragments bzw. eines Oligonukleotids).

dCTP

Desoxyribonucleosid-Triphosphat des C (DNA-Einheit mit der Base C und zwei weiteren Phosphaten zum Aufbau eines längeren DNA-Fragments bzw. eines Oligonukleotids).

dTTP

Desoxyribonucleosid-Triphosphat des T (DNA-Einheit mit der Base T und zwei weiteren Phosphaten zum Aufbau eines längeren DNA-Fragments bzw. eines Oligonukleotids).

Primer

Start-Komplementär-Fragment eines Oligonukleotids, wobei die Basenlänge des Primers nur ca. 4-8 Basen beträgt. Dient als Ansatzpunkt für die enzymatische Replikation des Oligonukleotids.

Mismatch

Zur Ausbildung der Watson Crick Struktur doppelsträngiger Oligonukleotide hybridisieren die beiden Einzelstränge derart, daß die Base A (bzw. C) des einen Strangs mit der Base T (bzw. G) des anderen Strangs Wasserstoffbrücken ausbildet (bei RNA ist T durch Uracil ersetzt). Jede andere Basenpaarung bildet keine Wasserstoffbrücken aus, verzerrt die Struktur und wird als "Mismatch" bezeichnet.

6

ds

double strand (Doppelstrang)

SS

single strand (Einzelstrang)

Chemische Substanzen/Gruppen

R beliebiger, nicht näher spezifizierter organischer Rest als Substituent

oder Seitenkette.

Redox redoxaktive Substanz

Alkyl Der Begriff "Alkyl" bezeichnet ein gesättigtes

Kohlenwasserstoffradikal, das geradkettig oder verzweigt ist (z.B. Ethyl, Isopropyl oder 2,5-Dimethylhexyl etc.). Wenn "Alkyl" benutzt wird, um auf einen Linker oder Spacer zu verweisen, bezeichnet der Begriff eine Gruppe mit zwei verfügbaren Valenzen für die kovalente Verknüpfung (z. B. -CH₂CH₂-, -CH₂CH₂-CH₂- oder -CH₂C(CH₃)₂CH₂CH₂C(CH₃)₂CH₂- etc.). Bevorzugte Alk irruppen als Substituenten oder Seitenketten R sind solche der Kettenlänge 1-30 (längste durchgehende Kette von aneinandergebundenen Atomen). Bevorzugte Alkylgruppen als Linker oder Spacer sind solche der Kettenlänge 1-20, insbesondere der Kettenlänge 1-14, wobei die Kettenlänge die kürzeste durchgehende Verbindung zwischen

den Linker oder Spacer verbundenen Strukturen darstellt.

Alkylgruppen bei denen eine oder mehrere der C-C Einfachbindungen durch C=C Doppelbindungen ersetzt sind.

Alkinyl Alkyl- oder Alkenylgruppen bei denen eine oder mehrere der

C-C Einfach- oder C=C Doppelbindungen durch C≡C

Dreifachbindungen ersetzt sind.

Hetero-Alkyl Alkylgruppen bei denen eine oder mehrere der C-H Bindungen

oder C-C Einfachbindungen durch C-N, C=N, C-P, C=P, C-O,

C=O, C-S oder C=S Bindungen ersetzt sind.

Hetero-Alkenyl Alkenylgruppen bei denen eine oder mehrere C-H Bindungen,

C-C Einfach- oder C=C Doppelbindungen durch C-N, C=N, C-P,

C=P, C-O, C=O, C-S oder C=S Bindungen ersetzt sind.

Hetero-Alkinyl Alkinylgruppen bei denen eine oder mehrere der C-H

Bindungen, C-C Einfach-, C=C Doppel- oder C=C Dreifachbindung durch C-N, C=N, C-P, C=P, C-O, C=O, C-S

oder C=S Bindungen ersetzt sind.

Alkenyl

Linker

molekulare Verbindung zwischen zwei Molekülen bzw. zwischen Oberflächenatom, Oberflächenmolekül oder einer Oberflächenmolekülgruppe und einem anderen Molekül. In der Regel sind Linker als Alkyl-, Alkenyl-, Alkinyl-, Hetero-Alkyl-, Hetero-Alkenyl- oder Heteroalkinylkette käuflich zu erwerben, wobei die Kette an zwei Stellen mit (gleichen oder verschiedenen) reaktiven Gruppen derivatisiert ist. Diese Gruppen bilden in einfachen/bekannten chemischen Reaktionen mit den entsprechenden Reaktionspartner eine kovalente chemische Bindung aus. Die reaktiven Gruppen können auch photoaktivierbar sein, d. h. die reaktiven Gruppen werden erst durch Licht bestimmter oder beliebiger Wellenlänge aktiviert. Bevorzugte Linker sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge hier die kürzeste durchgehende Verbindung zwischen den zu verbindenden Strukturen, also zwischen den zwei Molekülen bzw. zwischen einem Oberflächenatom, Oberflächenmolekül oder einer Oberflächenmolekülgruppe und einem anderen Molekül, darstellt.

Spacer

Linker, der über die reaktiven Gruppen an eine oder beide der zu verbindenden Strukturen (siehe Linker) kovalent angebunden ist. Bevorzugte Spacer sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge die kürzeste durchgehende Verbindung zwischen den zu verbindenden Strukturen darstellt.

(n x HS-Spacer)oligo Nukleinsäure-Oligomer, an das n Thiolfunktionen über jeweils einen Spacer angebunden sind, wobei die Spacer jeweils eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Thiolfunktion und Nukleinsäure-Oligomer) aufweisen können, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14. Diese Spacer können wiederum an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diesem durch Modifikation angebrachte reaktive Gruppen gebunden sein und "n" ist eine beliebige ganze Zahl, insbesondere eine Zahl zwischen 1 und 20.

(n x R-S-S-Spacer)-oligo

R-S-S- Nukleinsäure-Oligomer, an das n Disulfidfunktionen über jeweils einen Spacer angebunden sind, wobei ein beliebiger Rest R die Disulfidfunktion absättigt. Der Spacer zur Anbindung der

Disulfidfunktion an das Nukleinsäure-Oligomer kann jeweils eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Disulfidfunktion und Nukleinsäure-Oligomer) aufweisen, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14. Diese Spacer können wiederum an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diesem durch Modifikation angebrachte reaktive Gruppen gebunden sein. Der Platzhalter "n" ist eine beliebige ganze Zahl, insbesondere eine Zahl zwischen 1 und 20.

oligo-Spacer-S-S-Spacer-oligo

zwei gleiche oder verschiedene Nukleinsäure-Oligomere, die über eine Disulfid-Brücke miteinander verbunden sind, wobei die Disulfidbrücke über zwei beliebige Spacer an die Nukleinsäure-Oligomere angebunden ist und die beiden Spacer eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Disulfidbrücke und dem jeweiligen Nukleinsäure-Oligomer) aufweisen können, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14 und diese Spacer wiederum an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diese durch Modifikation angebrachte reaktive Gruppen gebunden sein können.

PQQ

Pyrrolo-Chinolino-Chinon, entspricht: 4,5-Dihydro-4,5-dioxo-1H-

pyrrolo-[2,3-f]-chinolin-2,7,9-tricarboxylsäure)

TEATFB

Tetraethylammonium-tetrafluoroborat

sulfo-NHS

N-Hydroxysulfosuccinimid

EDC

(3-Dimethylaminopropyl)-carbodiimid

HEPES

N-[2-Hydroxyethyl]piperazin-N'-[2-ethansulfonsäure]

Tris

Tris-(hydroxymethyl)-aminomethan

EDTA

Ethylendiamin-Tetraacetat (Natriumsalz)

Cystamin

(H₂N-CH₂-CH₂-S-)₂

modifizierte Oberflächen/Elektroden

Mica	Muskovit-Plättchen,	Trägermaterial	zum Auf	bringen	dünner
	Schichten.				
Au-S-ss-oligo-PQQ	Gold-Film auf Mica	mit kovalent au	ufgebrachter	Monola	yer aus
	derivatisiertem 12E	p Einzelstrang	Oligonukle	eotid (S	eauenz.

TAGTCGGAAGCA). Hierbei ist die endständigen Phosphatgruppe des Oligonukleotids am 3' Ende mit (HO-(CH₂)₂-S)₂ zum P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert, wobei die S-S Bindung homolytisch gespalten wird und je eine Au-S-R Bindung bewirkt. Die endständige Base Thymin am 5'- Ende des Oligonukleotids ist am C-5 Kohlenstoff mit -CH=CH-CO-NH-CH₂-CH₂-NH₂ modifiziert und dieser Rest wiederum ist über seine freie Aminogruppe durch Amidbildung mit einer Carbonsäuregruppe des PQQ verbunden.

Au-S-ds-oligo-PQQ

Au-S-ss-oligo-PQQ, welches mit dem zu ss-oligo (Sequenz: TAGTCGGAAGCA) komplementären Oligonukleotid hybridisiert vorliegt.

Elektrochemie

Ε

Elektrodenpotential, das an der Arbeitselektrode anliegt.

 E_0

Halbstufenpotential, Potential in der Mitte zwischen den Strom-Maxima für Oxidation und Reduktion einer in der Cyclovoltametrie reversiblen Elektrooxidation oder -reduktion.

i

Stromdichte (Strom pro cm² Elektrodenoberfläche)

Cyclovoltametrie

Aufzeichnung einer Strom/Spannungskurve. Hierbei wird das Potential einer stationären Arbeitselektrode zeitabhängig linear verändert, ausgehend von einem Potential bei dem keine Elektrooxidation oder -reduktion stattfindet bis zu einem Potential bei dem eine gelöste oder an die Elektrode adsorbierte Spezies oxidiert oder reduziert wird (also Strom fließt). Nach Durchlaufen des Oxidations- bzw. Reduktionsvorgangs, der in der Strom/Spannungskurve einen zunächst ansteigenden Strom und nach Erreichen eines Maximums einen allmählich abfallenden Strom erzeugt, wird die Richtung des Potentialvorschubs umgekehrt. Im Rücklauf wird dann das Verhalten der Produkte der Elektrooxidation oder -reduktion aufgezeichnet.

Amperometrie

Aufzeichnung einer Strom/Zeitkurve. Hierbei wird das Potential einer stationären Arbeitselektrode z. B. durch einen Potentialsprung auf ein Potential gesetzt, bei dem die Elektrooxidation oder -reduktion einer gelösten oder adsorbierten Spezies stattfindet und der fließende Strom wird in Abhängigkeit von der Zeit aufgezeichnet.

WO 00/31101

Die vorliegende Erfindung betrifft ein Nukleinsäure-Oligomer, das durch chemische Anbindung einer redoxaktiven Substanz modifiziert ist. Als Nukleinsäure-Oligomer wird im Rahmen der vorliegenden Erfindung eine Verbindung aus wenigstens zwei kovalent verbundenen Nukleotiden oder aus wenigstens zwei kovalent verbundenen Pyrimidin- (z. B. Cytosin, Thymin oder Uracil) oder Purin-Basen (z. B. Adenin oder Guanin), bevorzugt ein DNA-, RNA- oder PNA-Fragment, verwendet. In der vorliegenden Erfindung bezieht sich der Begriff Nukleinsäure auf ein beliebiges "Rückgrat" der kovalent verbundenen Pyrimidin- oder Purin-Basen, wie z. B. auf das Zucker-Phosphat Rückgrat der DNA, cDNA oder RNA, auf ein Peptid-Rückgrat der PNA oder auf analoge Rückgrat-Strukturen, wie z. B. ein Thio-Phosphat-, ein Dithio-Phosphat- oder ein Phosphoramid-Rückgrat. Wesentliches Merkmal einer Nukleinsäure im Sinne der vorliegenden Erfindung ist, daß sie natürlich vorkommende cDNA oder RNA sequenzspezifisch binden kann. Alternativ zu dem Begriff "Nukleinsäure-Oligomer" werden die Begriffe "(Sonden-) Oligonukleotid", "Nukleinsäure" oder "Oligomer" verwendet.

Die redoxaktive Substanz ist bei einem Potential φ selektiv oxidierbar und reduzierbar, wobei φ der Bedingung 2,0 V $\geq \varphi \geq$ - 2,0 V genügt. Das Potential bezieht sich hierbei auf die freie, unmodifizierte, redoxaktive Substanz in einem geeigneten Lösungsmittel, gemessen gegen Normalwasserstoffelektrode. Im Rahmen der vorliegenden Erfindung ist der Potentialbereich 1,7 V ≥ φ ≥ - 1.7 V bevorzugt, wobei der Bereich 1,4 V $\geq \phi \geq$ - 1,2 V besonders bevorzugt ist und der Bereich 0,9 V $\geq \phi \geq$ - 0,7 V, in dem die redoxaktive Substanz des Anwendungsbeispiels reduziert und reoxidiert wird, ganz besonders bevorzugt ist. Daneben betrifft die vorliegende Erfindung eine leitfähige Oberfläche, an die direkt oder indirekt (über einen Spacer) ein Nukleinsäure-Oligomer mit angebundener redoxaktiver Substanz chemisch gebunden ist. Außerdem betrifft die vorliegende Erfindung ein Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche, wobei ein modifiziertes Nukleinsäure-Oligomer auf eine leitfähige Oberfläche aufgebracht wird. Gemäß einem weiteren Aspekt betrifft die vorliegende Erfindung ein Verfahren, das die molekularer Strukturen, insbesondere elektrochemische Detektion die DNA-/RNA-/PNAelektrochemische Detektion von Fragmenten einer sequenzspezifische Nukleinsäure-Oligomer-Hybridisierung Probenlösung durch ermöglicht. Die Detektion der Hybridisierungsereignisse durch elektrische Signale ist einfache und kostengünstige Methode und ermöglicht einer eine batteriebetriebenen Variante eines Sequenziergeräts den Einsatz vor Ort.



11

Bindung einer redoxaktiven Substanz an ein Nukleinsäure-Oligomer

Voraussetzung für das erfindungsgemäße Verfahren ist die Bindung einer redoxaktiven Substanz an ein Nukleinsäure-Oligomer. Erfindungsgemäß kann dazu jede redoxaktive Substanz verwendet werden, solange sie bei einem Potential φ, das der Bedingung 2,0 V $\geq \varphi \geq$ - 2,0 V genügt, selektiv oxidierbar und reduzierbar ist. Das Potential bezieht sich hierbei auf die freie, unmodifizierte, redoxaktive Substanz in einem geeigneten Lösungsmittel, gemessen gegen Normalwasserstoffelektrode. Im Rahmen der vorliegenden Erfindung ist der Potentialbereich 1,7 V $\geq \varphi \geq$ - 1.7 V bevorzugt, wobei der Bereich 1,4 V $\geq \phi \geq$ - 1,2 V besonders bevorzugt ist und der Bereich 0.9 V $\geq \omega \geq$ - 0.7 V , in dem die redoxaktive Substanz des Anwendungsbeispiels reduziert und reoxidiert wird, ganz besonders bevorzugt ist. Unter dem Begriff "selektiv oxidierbar und reduzierbar" wird im Rahmen der vorliegenden Erfindung eine Redoxreaktion, also Abgabe oder Aufnahme eines Elektrons, verstanden, welche selektiv am Ort der redoxaktiven Substanz stattfindet. Durch das angelegte Potential wird also letztendlich kein anderer Teil des Nukleinsäure-Oligomers reduziert oder oxidiert, sondern ausschließlich die an das Nukleinsäure-Oligomer gebundene redoxaktive Substanz.

Unter redoxaktiver Substanz wird erfindungsgemäß jedes beliebige Molekül verstanden, daß im elektrochemisch zugänglichen Potentialbereich der jeweiligen Trägeroberfläche (Elektrode) durch Anlegen einer äußeren Spannung an dieser Elektrode elektrooxidiert/-reduziert werden kann. Neben üblichen organischen und anorganischen redoxaktiven Substanzen wie z. B. Hexacyanoferraten, Ferrocenen, Acridinen oder Phtalocyaninen eignen sich zur Anbindung an das Sonden-Oligonukleotid insbesondere redoxaktive Farbstoffe wie z. B. (Metallo-) Porphyrine der allgemeinen Formel 1, (Metallo-) Chlorophylle der allgemeinen Formel 2 oder (Metallo-) Bakteriochlorophylle der allgemeinen Formel 3, (farbige) natürlich vorkommende Oxidations-Agentien wie z. B. Flavine der allgemeinen Formel 4, Pyridin-Nukleotide der allgemeinen Formel 5 oder Pyrrolo-Chinolin-Chinone (PQQ) der allgemeinen Formel 6 oder sonstige Chinone wie z. B 1,4-Benzochinone der allgemeinen Formel 8, 1,4-Naphtochinone der allgemeinen Formel 9, 1,2-Naphtochinone der allgemeinen Formel 10 oder 9,10-Anthrachinone der allgemeinen Formel 11.

$$R_{12}$$
 R_{12}
 R_{13}
 R_{4}
 R_{10}
 R_{9}
 R_{8}
 R_{7}

Formel 1

$$R_{12}$$
 R_{12}
 R_{13}
 R_{14}
 R_{10}
 R

Formel 2

Formel 3

M = 2H, Mg, Zn, Cu, Ni, Pd, Co, Cd, Mn, Fe, Sn, Pt etc.; R_1 bis R_{12} sind unabhängig voneinander H oder beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.

$$R_2$$
 R_3
 R_4
 R_5

Formel 4

$$R_2$$
 R_3
 N^+
 R_5
 R_4

Formel 5

$$R_1$$
 R_2
 R_3
 R_4

Formel 6

$$R_1$$
 R_2
 R_3
 R_4

Formel 7

$$R_1$$
 R_2
 R_3
 R_3

Formel 8

$$R_2$$
 R_3
 R_4
 R_5
 R_6

Formel 9

$$R_2$$
 R_3
 R_4
 R_5

Formel 10

$$R_{2}$$

$$R_{3}$$

$$R_{4}$$

$$R_{5}$$

$$R_{6}$$

Formel 11

 R_1 bis R_8 sind unabhängig voneinander H oder beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.



Erfindungsgemäß wird eine redoxaktive Substanz an ein Oligonukleotid kovalent durch die Reaktion des Oligonukleotids mit der redoxaktiven Substanz gebunden. Diese Bindung kann auf drei verschiedene Arten durchgeführt werden:

- a) Als reaktive Gruppe zur Bindungsbildung am Nukleinsäure-Oligomer wird eine freie Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe des Oligonukleotid-Rückgrats, insbesondere eine Gruppe an einem der beiden Enden des Oligonukleotid-Rückgrats, verwendet. Die freien, endständigen Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen weisen eine erhöhte Reaktivität auf und gehen daher leicht typische Reaktionen wie z. B. Amidbildung mit (primären oder sekundären) Aminogruppen bzw. mit Säuregruppen, Esterbildung mit (primären, sekundären oder tertiären) Alkoholen bzw. mit Säuregruppen, Thioesterbildung mit (primären, sekundären oder tertiären) Thio-Alkoholen bzw. mit Säuregruppen oder die Kondensation von Amin und Aldehyd mit anschließender Reduktion der entstandenen CH=N Bindung zur CH₂-NH Bindung ein. Die zur kovalenten Anbindung der redoxaktiven Substanz nötige Kopplungsgruppe (Säure-, Amin-, Alkohol-, Thioalkohol- oder Aldehydfunktion) ist entweder natürlicherweise an der redoxaktiven Substanz vorhanden oder wird durch chemische Modifikation der redoxaktiven Substanz erhalten.
- b) Das Nukleinsäure-Oligomer ist über einen kovalent angebundenen Molekülteil (Spacer) beliebiger Zusammensetzung und Kettenlänge (kürzeste durchgehende Verbindung zwischen den zu verbindenden Strukturen darstellt), insbesondere der Kettenlänge 1 bis 14, am Oligonukleotid-Rückgrat bzw. an einer Base mit einer reaktiven Gruppe modifiziert. Die Modifikation erfolgt bevorzugt an einem der Enden des Oligonukleotid-Rückgrats bzw. an einer terminalen Base. Als Spacer kann z.B. ein Alkvl-. Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyloder Heteroalkinylsubstituent verwendet werden. Mögliche einfache Reaktionen zur Ausbildung der kovalenten Bindung zwischen redoxaktiver Substanz und des so modifizierten Nukleinsäure-Oligomers sind wie unter a) beschrieben, die Amidbildung aus Säure- und Amino-Gruppe, die Esterbildung aus Säure- und Alkohol-Gruppe, die Thioesterbildung aus Säure- und Thio-Alkohol-Gruppe oder die Kondensation von Aldehyd und Amin mit anschließender Reduktion der entstandenen CH=N Bindung zur CH₂-NH Bindung.

Gemäß einer bevorzugten Ausführungsform ist das Nukleinsäure-Oligomer durch eine redoxaktive Substanz modifiziert, die Bereiche mit einem überwiegend planaren, in einer Ebene ausgedehnten p- π -Orbital-System aufweist, wie z. B. das PQQ des Beispiels 1 oder die Chinone der Formel 5 oder 7-12 oder die porphinoiden

Strukturen der Formeln 1 - 4 bzw. die Pyridin-Nukleotide der allgemeinen Formel 6 bzw. Derivate dieser redoxaktiven Substanzen. In diesem Fall kann der Spacer, über den die redoxaktive Substanz an das Nukleinsäure-Oligomer gebunden ist, so gewählt werden, daß sich die Ebene der π -Orbitale der redoxaktiven Substanz parallel zu den p- π -Orbitalen der an die redoxaktive Substanz angrenzenden Basen des Nukleinsäure-Oligomers anordnen kann. Diese räumliche Anordnung von redoxaktiver Substanz mit teilweise planarem in einer Ebene ausgedehnten p- π -Orbitalen erweist sich als besonders günstig.

c) Bei der Synthese des Nukleinsäure-Oligomers wird eine terminale Base durch die redoxaktive Substanz ersetzt.

Erfindungsgemäß kann die Bindung der redoxaktiven Substanz an das Oligonukleotid wie unter a) und b) beschrieben vor oder nach der Bindung des Oligonukleotids an die leitfähige Oberfläche erfolgen. Die Anbindung der redoxaktiven Substanz an das auf der leitfähigen Oberfläche gebundene Oligonukleotid erfolgt dann ebenfalls wie unter a) und b) beschrieben.

Bei mehreren verschiedenen Oligonukleotid-Kombinationen (Test-Sites) auf einer gemeinsamen Oberfläche ist es vorteilhaft, die (kovalente) Anbindung der redoxaktiven Substanz an die Sonden-Oligonukleotide durch geeignete Wahl der reaktiven Gruppe an den freien Sonden-Oligonukleotidenden der verschiedenen Test-Sites für die gesamte Oberfläche zu vereinheitlichen.

Die leitfähige Oberfläche

Unter dem Begriff "leitfähige Oberfläche" wird erfindungsgemäß jeder Träger mit einer elektrisch leitfähigen Oberfläche beliebiger Dicke verstanden, insbesondere Oberflächen aus Platin, Palladium, Gold, Cadmium, Quecksilber, Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium und Mangan. Im Rahmen der vorliegenden Erfindung werden die Begriffe "Elektrode" und "leitfähige (Träger-) Oberfläche" alternativ zu "leitfähige Oberfläche "gebraucht.

Daneben können auch beliebige dotierte oder nicht dotierte Halbleiteroberflächen beliebiger Dicke verwendet werden. Sämtliche Halbleiter können als Reinsubstanzen oder als Gemische Verwendung finden. Als nicht einschränkend gemeinte Beispiele seien an dieser Stelle Kohlenstoff, Silizium, Germanium, α -Zinn, Cu(I)- und Ag(I)- Halogenide beleibiger Kristallstruktur genannt. Geeignet sind ebenfalls sämtliche

binären Verbindungen beliebiger Zusammensetzung und beliebiger Struktur der Elemente der Gruppen 14 und 16, der Elemente der Gruppen 13 und 15, sowie der Elemente der Gruppen 15 und 16. Daneben können ternäre Verbindungen beliebiger Zusammensetzung und beliebiger Struktur der Elemente der Gruppen 11, 13 und 16 oder der Elemente der Gruppen 12, 13 und 16 verwendet werden. Die Bezeichnungen der Gruppen des Periodensystems der Elemente beziehen sich auf die IUPAC-Empfehlung von 1985.

Bindung eines Oligonukleotids an die leitfähige Oberfläche

Erfindungsgemäß wird ein Oligonukleotid direkt oder über einen Linker/Spacer mit den Trägeroberflächenatomen oder -molekülen einer leitfähigen Trägeroberfläche der oben beschriebenen Art verknüpft. Diese Bindung kann auf drei verschiedene Arten durchgeführt werden:

a) Die Oberfläche wird so modifiziert, daß eine reaktive Molekül-Gruppe zugänglich ist. Dies kann durch direkte Derivatisierung der Oberflächenmoleküle, z. B. durch naßchemische oder elektrochemische Oxidation/Reduktion geschehen. So kann z. B. die Oberfläche von Graphitelektroden durch Oxidation naßchemisch mit Aldehydoder Carbonsäure-Gruppen versehen werden. Elektrochemisch besteht z. B. die Möglichkeit durch Reduktion in Gegenwart von Aryl-Diazoniumsalzen das entsprechende (funktionalisierte, also mit einer reaktiven Gruppe versehene) Aryl-Radikal oder durch Oxidation in Gegenwart von R'CO₂H das (funktionalisierte) R'-Radikal auf der Graphit-Elektrodenoberfläche anzukoppeln. Ein Beispiel der direkten Modifikation von Halbleiteroberflächen ist die Derivatisierung von Siliziumoberflächen zu reaktiven Silanolen, d. h. Silizium-Träger mit Si-OR" Gruppen an der Oberfläche. wobei R" ebenso wie R' einen beliebigen, funktionalisierten, organischen Rest darstellt (z.B. Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinylsubstituent). Alternativ kann die gesamte Oberfläche durch die kovalente Anbindung einer reaktiven Gruppe eines bifunktionalen Linkers modifiziert werden, so daß auf der Oberfläche eine monomolekulare Schicht beliebiger Moleküle entsteht, die, bevorzugt endständig, eine reaktive Gruppe enthalten. Unter dem Begriff "bifunktionaler Linker" wird jedes Molekül beliebiger Kettenlänge, insbesondere der Kettenlängen 2-14, mit zwei gleichen (homo-bifunktional) oder zwei verschiedenen (hetero-bifunktional) reaktiven Molekül-Gruppen verstanden.

Sollen mehrere verschiedene Test-Sites auf der Oberfläche durch Ausnutzen der Methodik der Photolithographie gebildet werden, so ist mindestens eine der

Gruppen des reaktiven homooder hetereo-bifunktionalen Linkers photoinduzierbar reaktive Gruppe, d. h. eine erst durch Lichteinstrahlung bestimmter oder beliebiger Wellenlänge reaktiv werdende Gruppe. Dieser Linker wird so aufgebracht, daß die/eine photoaktivierbare reaktive Gruppe nach der kovalenten Anbindung des Linkers auf der Oberfläche zur Verfügung steht. An die so modifizierte Oberfläche werden die Nukleinsäure-Oligomere kovalent angebunden, wobei diese selbst über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, mit einer reaktiven Gruppe modifiziert sind, bevorzugt in der Nähe eines Endes des Nukleinsäure-Oligomers. Bei der reaktiven Gruppe des Oligonukleotids handelt es sich um Gruppen, die direkt (oder indirekt) mit der modifizierten Oberfläche unter Ausbildung einer kovalenten Bindung reagieren. Daneben kann an die Nukleinsäure-Oligomere in der Nähe ihres zweiten Endes eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die redoxaktive Substanz alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Nukleinsäure-Orgamers angebunden sein.

b) Das Nukleinsäure-Oligomer, das auf die leitfähige Oberfläche aufgebracht werden soll, ist über einen kovalent angebundenen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, mit einer oder mehreren reaktiven Gruppe modifiziert, wobei sich diese reaktiven Gruppen bevorzugt in der Nähe eines Endes des Nukleinsäure-Oligomers befinden. Bei den reaktiven Gruppen handelt es sich um Gruppen, die direkt mit der unmodifizierten Oberfläche reagieren können. Beispiele hierfür sind: (i) Thiol- (HS-) oder Disulfid- (S-S-) derivatisierte Nukleinsäure-Oligomere der allgemeinen Formel (n x HS-Spacer)-oligo, (n x R-S-S-Spacer)-oligo oder oligo-Spacer-S-S-Spacer-oligo, die mit einer Goldoberfläche unter Ausbildung von Gold-Schwefelbindungen reagieren oder (ii) Amine, die sich durch Chemi- oder Physisorption an Platin- oder Silizium-Oberflächen anlagern. Daneben kann an die Nukleinsäure-Oligomere in der Nähe ihres zweiten Endes eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die redoxaktive Substanz alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Oligonukleotids angebunden sein. Insbesondere Nukleinsäure-Oligomere die mit mehreren Spacer-verbrückten Thiol oder Disulfidbrücken modifiziert sind ((n x HS-Spacer)-oligo bzw. (n x R-S-S-Spacer)oligo) haben den Vorteil, daß solche Nukleinsäure-Oligomere unter einem bestimmten Anstellwinkel gegen die leitfähige Oberfläche (Winkel zwischen der Oberflächennormalen und der Helixachse eines doppelsträngigen helikalen Nukleinsäure-Oligomers bzw. zwischen der Oberflächennormalen und der Achse senkrecht zu den Basenpaaren eines doppelsträngigen nicht-helikalen Nukleinsäure-Oligomers) aufgebracht werden können, wenn die die Thiol- bzw. Disulfid-Funktionen an das Nukleinsäure-Oligomer anbindenden Spacer, von einem Ende der Nukleinsäure her betrachtet, eine zunehmende bzw. abnehmende Kettenlänge besitzen.

reaktive Gruppe am Sonden-Nukleinsäure-Oligomer werden Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen des Oligonukleotid-Rückgrats, insbesondere endständige Gruppen, verwendet. Die Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen weisen eine erhöhte Reaktivität auf und gehen daher leicht typische Reaktionen wie z. B. Amidbildung mit (primären oder sekundären) Amino- bzw. Säuregruppen, Esterbildung mit (primären, sekundären oder tertiären) Alkoholen Säuregruppen, Thioesterbildung mit (primären, sekundären oder tertiären) Thio-Alkoholen bzw. Säuregruppen oder die Kondensation von Amin und Aldehyd mit anschließender Reduktion der entstandenen CH=N Bindung zur CH₂-NH Bindung ein. Die nötige Kopplungs-Gruppe zur kovalenten Anbindung an die Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe ist in diesem Fall ein Teil der Oberflächenderivatisierung mit einer (monomolekularen) Schicht beliebiger Moleküllänge, wie unter a) in diesem Abschnitt beschrieben, oder die Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe kann direkt mit der unmodifizierten Oberfläche reagieren, wie unter b) in diesem Abschnitt beschrieben. Daneben kann an die Oligonukleotide in der Nähe ihres zweiten Endes eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die redoxaktive Substanz alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Nukleinsäure-Oligomers angebunden sein.

Die Bindung des Oligonukleotids an die leitfähige Oberfläche kann alternativ vor oder nach der Anbindung der redoxaktiven Substanz an das Oligonukleotid bzw. vor oder nach Anbinden des mit einer reaktiven Gruppe versehenen Spacers zur Bindung der redoxaktiven Substanz erfolgen. Die Bindung des bereits modifizierten Oligonukleotids an die leitfähige Oberfläche, d. h. die Bindung an die Oberfläche nach der Anbindung der redoxaktiven Substanz an das Oligonukleotid bzw. nach der

Anbindung des mit einer reaktiven Gruppe versehenen Spacers zur Bindung der redoxaktiven Substanz, erfolgt ebenfalls wie unter a) bis c) (Abschnitt "Bindung eines Oligonukleotids an die leitfähige Oberfläche") beschrieben.

Bei der Herstellung der Test-Sites muß bei der Anbindung der Einzelstrang-Oligonukleotide an die Oberfläche darauf geachtet werden, daß zwischen den einzelnen Oligonukleotiden ein genügend großer Abstand verbleibt, um den für eine Hybridisierung mit dem Target-Oligonukleotid nötigen Freiraum zur Verfügung zu stellen. Dazu bieten sich unter anderem zwei verschiedene Vorgehensweisen an:

- 1.) Herstellung einer modifizierten Trägeroberfläche durch Anbindung eines hybridisierten Oligonukleotids, also eine Trägeroberflächen-Derivatisierung mit hybridisiertem Sonden-Oligonukleotid statt mit Einzelstrang-Sonden-Oligonukleotid. Der zur Hybridisierung verwendete Oligonukleotidstrang ist unmodifiziert (die Oberflächenanbindung wird durchgeführt wie unter a) c) im Abschnitt "Bindung eines Oligonukleotids an die leitfähige Oberfläche" beschrieben). Anschließend wird der hybridisierte Oligonukleotid-Doppelstrang thermischer dehybridisiert, wodurch eine Einzelstrang-Oligonukleotid modifizierte Trägeroberfläche mit größerem Abstand zwischen den Proboligonukleotiden hergestellt wird.
- 2.) Herstellung einer modifizierten Trägeroberfläche durch Anbindung eines Einzelstrangoder Doppelstrang-Oligonukleotids, wobei während der Trägeroberflächen-Derivatisierung ein geeigneter monofunktionaler Linker zugesetzt wird, der neben dem Einzelstrang- oder Doppelstrang-Oligonukleotid auch an die Oberfläche gebunden wird (die Oberflächenanbindung wird durchgeführt wie unter a) - c) im Abschnitt "Bindung eines Oligonukleotids an die leitfähige Oberfläche" beschrieben). Erfindungsgemäß hat der monofunktionale Linker eine Kettenlänge, die der Kettenlänge des Spacers zwischen Trägeroberfläche und Oligonukleotid identisch ist oder um maximal acht Kettenatome abweicht. Bei der Verwendung von Doppelstrang-Oligonukleotid zur Trägeroberflächen-Derivatisierung wird nach der Anbindung des Doppelstrang-Oligonukleotids und des Linkers an die Trägeroberfläche der hybridisierte Oligonukleotid-Doppelstrang thermischer dehybridisiert, wie oben unter 1.) beschrieben. Durch die gleichzeitige Anbindung eines Linkers an die Oberfläche wird der Abstand zwischen den ebenfalls an die Oberfläche gebundenen Einzel- oder Doppelstrang-Nukleinsäure-Oligomeren vergrößert. Im Falle der Verwendung von Doppelstrang-Nukleinsäure-Oligomer wird dieser Effekt durch die anschließende thermische Dehybridisierung noch verstärkt.

Verfahren zur elektrochemischen Detektion von Nukleinsäureoligomerhybriden

20

Vorteilhafterweise werden gemäß dem Verfahren zur elektrochemischen Detektion mehrere Sonden-Oligonukleotide unterschiedlicher Sequenz, idealerweise alle nötigen Kombinationen des Nukleinsäure-Oligomers, auf einem Oligomer- oder DNA-Chip aufgebracht, um die Sequenz eines beliebigen Target-Oligomers oder einer (fragmentierten) Target-DNA sicher zu detektieren bzw. um Mutationen im Target aufzuspüren und sequenzspezifisch nachzuweisen. Dazu werden auf einer leitfähigen Trägeroberfläche die Trägeroberflächenatome oder -moleküle eines definierten Bereichs (einer Test-Site) mit DNA-/RNA-/PNA-Oligonukleotiden bekannter, aber beliebiger Sequenz, wie oben beschrieben, verknüpft. In einer allgemeinsten Ausführungsform kann aber der DNA-Chip auch mit einem einzigen Sonden-Oligonukleotid derivatisiert werden. Als Sonden-Oligonukleotide werden Nukleinsäure-Oligomere (DNA-, RNA- oder PNA-Fragmente) der Basenlänge 3 bis 50, bevorzugt der Länge 5 bis 30, besonders bevorzugt der Länge 7 bis 25 verwendet. Erfindungsgemäß wird an die Sonden-Oligonukleotide entweder vor oder nach deren Bindung an die leitfähige Oberfläche eine redoxaktive Substanz gebunden.

Erfolgt die Modifikation der Sonden-Oligonukleotide vor der Bindung an die leitfähige Oberfläche, so werden die bereits modifizierten Sonden-Oligonukleotide wie oben beschrieben an die leitfähige Oberfläche gebunden. Alternativ werden die nicht modifizierten, an die leitfähige Oberfläche gebundenen Sonden-Oligonukleotide am zweiten, freien Ende der Oligonukleotidkette direkt oder indirekt über einen Spacer mit einer redoxaktive Substanz modifiziert.

In beiden Fällen entsteht ein Oberflächen-Hybrid der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Redox (Figur 3). Die elektrische Kommunikation zwischen der (leitfähigen) Trägeroberfläche und dem über ein Einzelstrang-Oligonukleotid verbrückten redoxaktiven Substanz ("Redox") in der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Redox ist schwach oder gar nicht vorhanden. Die Verbrückungen können natürlich auch ohne Spacer oder mit nur einem Spacer (Elek-ss-oligo-Spacer-Redox bzw Elek-Spacer-ss-oligo-Redox) durchgeführt werden.

In einem nächsten Schritt werden die Test-Sites mit der zu untersuchenden Oligonukleotid-Lösung (Target) in Kontakt gebracht. Dabei kommt es nur in dem Fall zur Hybridisierung, in dem die Lösung Oligonukleotid-Stränge enthält, die zu den an die leitfähige Oberfläche gebundenen Sonden-Oligonukleotiden komplementär, oder zumindest in weiten Bereichen komplementär sind. Im Falle der Hybridisierung zwischen Sonden- und Target-Oligonukleotid kommt es zu einer verstärkten Leitfähigkeit zwischen der Trägeroberfläche und der redoxaktiven Substanz, da

diese nunmehr über das aus einem Doppelstrang bestehende Oligonukleotid verbrückt sind (in Figur 3 an einem Beispiel der Elek-Spacer-ss-oligo-Spacer-Redox schematisch gezeigt).

Durch die Veränderung der elektrischen Kommunikation zwischen der (leitfähigen) Trägeroberfläche und der redoxaktiven Substanz aufgrund der Hybridisierung von Sonden-Oligonukleotid und dem dazu komplementären Oligonukleotid-Strang (Target) kann somit ein sequenzspezifisches Hybridisierungsereignis durch elektrochemische Verfahren wie z. B. Cyclovoltametrie, Amperometrie oder Leitfähigkeitsmessungen detektiert werden.

In einer besonders bevorzugten Ausführungsform der vorliegenden Erfindung wird eine redoxaktive Substanz verwendet, die Bereiche mit einem überwiegend planaren, in einer Ebene ausgedehnten p- π -Orbital-System aufweist, wie z. B. das PQQ des Beispiels 1 (vgl. Figur 3), oder die Chinone der Formel 5 oder 7-12 oder die porphinoiden Strukturen der Formeln 1 - 4, die Pyridin-Nukleotide der allgemeinen Formel 6 sowie Derivate dieser redoxaktiven Substanzen. In diesem Fall wird der Spacer zwischen Nukleinsäure-Oligomer und der redoxaktiven Substanz so gewählt, daß sich die Ebene der π -Orbitale der redoxaktiven Substanz parallel zu den p- π -Orbitalen des an die redoxaktive Substanz angrenzenden Basenpaars des mit Komplimentärstrang hybridisierten Nukleinsäure-Oligomers anordnen kann. Diese räumliche Anordnung von redoxaktiver Substanz mit teilweise planarem in einer Ebene ausgedehnten p- π -Orbitalen erweist sich für die elektrische Leitfähigkeit der Doppelstrang-Nukleinsäure-Oligomere als besonders günstig.

Bei der Cyclovoltametrie wird das Potential einer stationären Arbeitselektrode zeitabhängig linear verändert. Ausgehend von einem Potential bei dem keine Elektrooxidation oder reduktion stattfindet, wird das Potential solange verändert bis die redoxaktive Substanz oxidiert oder reduziert wird (also Strom fließt). Nach Durchlaufen des Oxidations- bzw. Reduktionsvorgangs, der in der Strom/Spannungskurve einen zunächst ansteigenden Strom, einen Maximalstrom (Peak) und dann einen allmählich abfallenden Strom erzeugt, wird die Richtung des Potentialvorschubs umgekehrt. Im Rücklauf wird dann das Verhalten der Produkte der Elektrooxidation oder -reduktion aufgezeichnet.

Eine alternative elektrische Detektionsmethode, die Amperometrie, wird dadurch ermöglicht, daß die redoxaktive Substanz durch Anlegen eines geeigneten, konstant gehaltenen Elektrodenpotentials zwar elektrooxidiert (elektroreduziert) wird, die Rereduktion (Reoxidation) der redoxaktiven Substanz in den ursprünglichen Zustand aber nicht durch Änderung des Elektrodenpotentials erreicht wird, wie in der

Cyclovoltametrie, sondern durch ein der Targetlösung zugesetztes geeignetes Reduktionsmittel (Oxidationsmittel), wodurch der Stromkreis des Gesamtsystems geschlossen wird. Solange Reduktionsmittel (Oxidationsmittel) vorhanden ist bzw. solange verbrauchtes Reduktionsmittel (Oxidationsmittel) an der Gegenelektrode rereduziert (reoxidiert) wird, fließt Strom, der amperometrisch detektiert werden kann und der proportional zur Zahl der Hybridisierungsereignisse ist.

Kurze Beschreibung der Zeichnungen

Die Erfindung soll nachfolgend anhand eines Ausführungsbeispiels im Zusammenhang mit den Zeichnungen näher erläutert werden. Es zeigen

- Fig. 1 Schematische Darstellung des Sanger-Verfahrens der Oligonukleotid-Sequenzierung;
- Fig. 2 Schematische Darstellung der Oligonukleotid-Sequenzierung durch Hybridisierung auf einem Chip;
- Schematische Darstellung des Oberflächen-Hybrids der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Redox mit einem 12 Bp Sonden-Oligonukleotid der exemplarischen Sequenz 5'-TAGTCGGAAGCA-3' (links) und Au-S-ss-oligo-PQQ im hybridisierten Zustand als Ausführungsbeispiel einer Elek-Spacer-ss-oligo-Spacer-Redox, wobei nur ein Teil des Sonden-Oligonukleotids mit hybridisierten Komplementärstrang gezeigt ist (rechts) und die Anbindung des Oligonukleotids an die Oberfläche über einen -S-CH₂CH₂- Spacer sowie die Anbindung der redoxaktiven Substanz PQQ über den Spacer -CH₂-CH=CH-CO-NH-CH₂-CH₂-NH- erfolgte;
- Fig. 4 Cyclovoltagramm einer Test-Site aus Au-S-ss-oligo-PQQ (gepunktet) im Vergleich zu einer identischen Test-Site mit vollständig hybridisiertem Target (Au-S-ds-oligo-PQQ, durchgezogene Linie);
- Fig. 5 Cyclovoltagramm einer Test-Site mit vollständig hybridisiertem Target (Au-S-ds-oligo-PQQ) (durchgezogene Linie) im Vergleich zu einer Test-Site mit hybridisiertem Target, das 2 Basenpaar Mismatches aufweist (Au-S-ds-oligo-PQQ mit 2 Bp Mismatches, gestrichelt).

23

Wege zur Ausführung der Erfindung

Eine exemplarische Test-Site mit hybridisiertem Target (Au-S-ds-oligo-PQQ) der allgemeinen Struktur Elek-Spacer-ds-oligo-Spacer-Redox ist in Figur 3 dargestellt. In dem Beispiel der Figur 3 ist die Trägeroberfläche eine Gold-Elektrode. Die Verbindung zwischen Gold-Elektrode und Sonden-Oligonukleotid wurde mit dem Linker (HO-(CH₂)₂-S)₂ aufgebaut, der mit der endständigen Phosphatgruppe am 3' Ende zu P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert wurde und nach homolytischer Spaltung der S-S Bindung an der Gold-Oberfläche je eine Au-S Bindung bewirkte, womit 2-Hydroxymercaptoethanol und Mercaptoethanol-verbrücktes Oligonukleotid auf der Oberfläche koadsorbiert wurde. Die redoxaktive Substanz im Beispiel der Figur 3 ist Pyrrolo-Chinolin-Chinon (PQQ), wobei eine der drei tricarboxylisches Carbonsäurefunktionen des PQQ (im Beispiel die C-7-CO₂H-Funktion) zur kovalenten Anbindung des PQQ an das Sonden-Oligonukleotid verwendet wurde (Amidbildung unter Wasserabspaltung mit der terminalen Aminofunktion des an die C-5-Position des 5'-Thymins angebundenen -CH=CH-CO-NH-CH₂-CH₂-NH₂ Spacers). Sowohl freies, unmodifiziertes PQQ als auch über einen kurzen Spacer der Kettenlänge 1-6, wie z. B. -S-(CH₂)₂-NH-, oder über (modifiziertes) Doppelstrang-Oligonukleotid mit der Trägeroberfläche verbrücktes PQQ wird, z. B. in HEPES Puffer mit 0.7 molarem Zusatz von TEATFB (siehe Abkürzungen), im Potentialbereich 0,7 V $\geq \phi \geq$ 0,0 V, gemessen gegen Normalwasserstoffelektrode, selektiv reduziert und reoxidiert.

Die elektrische Kommunikation zwischen der (leitfähigen) Trägeroberfläche und dem über Einzelstrang-Oligonukleotid verbrückten Redoxpaar in der allgemeinen Struktur Elek-Spacer-ds-oligo-Spacer-Redox ist schwach oder gar nicht vorhanden. Für die exemplarische Test-Site Au-S-ss-oligo-PQQ (mit 12-Bp Sonden-Oligonukleotiden) ist dies arihand der Cyclovoltametrie (Figur 4) gezeigt. Ohne an eine theoretische Beschreibung gebunden sein zu wollen, wird angenommen, daß die negativen Ladungen des Phosphatgerüsts eine gegenseitig Abstoßung der Oligonukleotid-Einzelstränge bedingen und so einen Aufbau der -Spacer-ds-oligo-Spacer-Redox Kette (in Richtung Helixachse) unter einem Winkel ϕ < 70 ° zur Normalen der Trägeroberfläche ("stehende Röhren") erzwingen. Die (hybridisierte) Test-Site Au-S-ds-oligo-PQQ der Figur 3 weist einen Aufbau mit ϕ = 30 ° auf. Aufgrund der Länge der -Spacer-ds-oligo-Spacer-Redox Kette (z. B. ca. 40 Å Länge eines 12-Basenpaar-Oligonukleotids; die Spacer und das angebundene PQQ sind rund 10 Å lang)

WO 00/31101 PCT/EP99/08888

24

entsteht bei ϕ < 70 ° zwischen Trägeroberfläche und redoxaktiver Substanz ein Abstand von > 17 Å. Dadurch kann ein direkter Elektron- oder Lochtransfer zwischen Trägeroberfläche und redoxaktiver Substanz ausgeschlossen werden. Durch Behandlung der Test-Site(s) mit einer zu untersuchenden Oligonukleotid-Lösung, kommt es, im Falle der Hybridisierung zwischen Sonde und Target zu einer verstärkten Leitfähigkeit zwischen der Trägeroberfläche und dem über das Doppelstrang-Oligonukleotid verbrückten Redoxpaar. Die Änderung der Leitfähigkeit äußert sich cyclovoltammetrisch in einem deutlichen Stromfluß zwischen Trägeroberfläche und redoxaktiver Substanz (Figur 4). Damit ist es möglich, die sequenzspezifische Hybridisierung des Targets mit den Sonden-Oligonukleotiden durch elektrochemische Verfahren wie z. B. cyclische Voltametrie zu detektieren.

Daneben können fehlerhafte Basenpaarungen (Basenpaar Mismatches) durch eine geänderte cyclovoltammetrische Charakteristik erkannt werden (Figur 5). Ein Mismatch äußert sich in einem größeren Potentialabstand zwischen den Strommaxima der Elektoreduktion und der Elektroreoxidation (Umkehrung der Elektroreduktion bei um gekehrter Potentialvorschubrichtung) bzw. der Elektrooxidation und Elektrorereduktion in einem cyclovoltammetrisch reversiblen Elektronenübertragungsprozess zwischen der elektrisch leitenden Trägeroberfläche und der redoxaktiven Substanz. Dieser Umstand wirkt sich vor allem in der amperometrischen Detektion günstig aus, da dort der Strom bei einem Potential getestet werden kann, bei dem zwar das perfekt hybridisierende Oligonukleotid-Target signifikant Strom liefert, nicht aber das fehlerhaft gepaarte Oligonukleotid-Target. Im Beispiel der Figur 5 ist dies bei einem Potential E-E₀ von ca. 0,03 V möglich.

Beispiel 1: Herstellung der Oligonukleotid-Elektrode Au-S-ds-oligo-PQQ. Die Herstellung von Au-S-ds-oligo-PQQ gliedert sich in 4 Teilabschnitte, nämlich Darstellung der Trägeroberfläche, Hybridisierung des Sonden-Oligonukleotids mit dem komplementären Doppelstrang (Hybridisierungsschritt), Derivatisierung der Trägeroberfläche mit dem Doppelstrang-Oligonukleotid (Inkubationsschritt) und Anbindung der redoxaktiven Substanz (Redoxschritt).

Das Trägermaterial für die kovalente Anbindung der Doppelstrang-Oligonukleotide bildet ein ca. 100nm dünner Gold-Film auf Mica (Muskovit Plättchen). Dazu wurde in einer elektrischen Entladungskammer frisch gespaltenes Mica mit einem Argon-Ionenplasma gereinigt und durch elektrische Entladung Gold (99.99%) in einer Schichtdicke von ca. 100nm aufgebracht. Anschließend wurde der Gold-Film mit 30 % $\rm H_2O_2$, / 70 % $\rm H_2SO_4$ von Oberflächenverunreinigungen befreit (Oxidation organischer Ablagerungen) und für ca. 20 Minuten in Ethanol getaucht, um an der

Oberfläche adsorbierten Sauerstoff zu verdrängen. Nach Abspülen der Trägeroberfläche mit bidestilliertem Wasser wird auf die horizontal gelagerte Trägeroberfläche eine vorher bereitete 1 x 10⁻⁴ molare Lösung des (modifizierten) Doppelstrang-Oligonukleotids aufgetragen, so daß die komplette Trägeroberfläche benetzt wird (Inkubationsschritt, siehe auch unten).

Zur Bereitung der ds-Oligonukleotid Lösung wurde ein doppelt modifiziertes 12 Bp Einzelstrang-Oligonukleotid der Sequenz 5'-TAGTCGGAAGCA-3' verwendet, das an der Phosphatgruppe des 3' Endes mit $(HO-(CH_2)_2-S)_2$ zum P-O- $(CH_2)_2-S-S-(CH_2)_2-OH$ verestert ist. Am 5'-Ende ist die endständige Base des Oligonukleotids, Thymin, am C-5 Kohlenstoff mit -CH=CH-CO-NH-CH₂-CH₂-NH₂ modifiziert. Eine 2 x 10⁻⁴ Lösung dieses Oligonukleotids im Hybridisierungspuffer (10 mM Tris, 1 mM EDTA, pH 7.5 mit 0.7 molarem Zusatz von TEATFB, siehe Abkürzungen) wurde mit einer 2 x 10-4 molaren Lösung des (unmodifizierten) komplementären Strang Hybridisierungspuffer bei Raumtemperatur für ca. 2h hybridisiert (Hybridisierungsschritt). Während einer Reaktionszeit von ca. 12-24h wurde der Disulfidspacer P-O-(CH₂)₂-S-S-(CH₂)₂-OH des Oligonukleotids homolytisch gespalten. Dabei bildet der Spacer mit den Au-Atomen der Oberfläche eine kovalente Au-S Bindung aus, wodurch es zu einer 1:1 Koadsorption des ds-Oligonukleotids und des 2-Hydroxy-mercaptoethanols kommt (Inkubationsschritt).

Die so mit einer dichten (1:1) Monolayer aus ds-Oligonukleotid und 2-Hydroxymercaptoethanol modifizierte Goldelektrode wurde mit bidestilliertem Wasser gewaschen und anschließend mit einer Lösung von 3x10⁻³ molarem Chinon PQQ, 10⁻² molarem EDC und 10⁻² molarem sulfo-NHS in HEPES Puffer benetzt. Nach einer Reaktionszeit von ca. 1h bindet der -CH=CH-CO-NH-CH₂-CH₂-NH₂ Spacer das PQQ kovalent an (Amidbildung zwischen der Aminogruppe des Spacers und einer Säurefunktion des PQQ, Redoxschritt).

Die Aufklärung der Oberflächenbeschaffenheit mit XPS (X-Ray Photoelektronenspektroskopie) ergab eine maximal dicht gepackte Monolayer aus 1:1 koadsorbiertem ds-Oligonukleotid und 2-Hydroxy-mercaptoethanol (4.7 x 10^{12} ds-Oligonukleotide/cm²), wobei die lange Achse (Richtung der Helixachse) der ds-Oligonukleotide mit der Flächennormalen der Goldoberfläche einen Winkel von $\phi \approx 30$ ° bildet.

Beispiel 2: Herstellung der Oligonukleotid-Elektrode Au-S-ss-oligo-PQQ. Analog zur Darstellung des Systems Au-S-ds-oligo-PQQ wird die Trägeroberfläche mit modifiziertem Einzelstrang-Oligonukleotid derivatisiert, wobei lediglich auf die

WO 00/31101 PCT/EP99/08888

26

Hybridisierung des modifizierten Oligonukleotids der Sequenz 5'-TAGTCGGAAGCA-3' mit seinem komplementären Strang verzichtet wurde und im Inkubationsschritt nur das doppelt modifizierte 12 Bp Einzelstrang-Sonden-Oligonukleotid (siehe Beispiel 1) als 1 x 10⁻⁴ molare Lösung in Wasser und in Gegenwart von 10⁻² molarem Tris, 10⁻³ molarem EDTA und 0.7 molarem TEATFB (oder 1 molarem NaCl) bei pH 7.5 verwendet wurde. Der Redoxschritt wurde, wie in Beispiel 1 angegeben, durchgeführt.

Beispiel 3: Herstellung der Oligonukleotid-Elektrode Au-S-ds-oligo-PQQ mit 2-Bp-Mismatches. Die Herstellung einer Trägeroberfläche derivatisiert mit modifiziertem Doppelstrang-Oligonukleotid wurde analog zur Darstellung des Systems Au-S-ds-oligo-PQQ durchgeführt, wobei lediglich bei der Hybridisierung des modifizierten Oligonukleotids der Sequenz 5'-TAGTCGGAAGCA-3' ein komplementärer Strang verwendet wurde (Sequenz: 5'-ATCAGATTTCGT-3'), bei dem die eigentlich komplementären Basen Nr. 6 und 7 (vom 5'-Ende gezählt) von C nach A bzw. von C nach T modifiziert wurden, um so zwei Basenpaar Mismatches einzuführen.

Beispiel 4: Herstellung einer Oligonukleotid-Elektrode Au-S-ss-oligo-PQQ mit erhöhtem Inter-Oligonukleotid-Abstand. Bei der Herstellung der Test-Sites muß bei der Derivatisierung der Trägeroberfläche mit Einzelstrang-Sonden-Oligonukleotid darauf geachtet werden, daß zwischen den angebundenen Einzelsträngen genügend Platz verbleibt, um eine Hybridisierung mit dem Target-Oligonukleotid zu ermöglichen. Dazu bieten sich drei verschiedene Vorgehensweisen an: (a) Herstellung einer Au-S-ds-oligo-PQQ Elektrode wie in Beispiel 1 beschrieben mit anschließender thermischer Dehybridisierung der Doppelstränge bei Temperaturen von T > 40 °C. (b) Herstellung einer Au-ss-oligo-PQQ Elektrode wie unter Beispiel 2 beschrieben, aber im Inkubationsschritt zur Derivatisierung der Goldoberfläche mit (doppelt Einzelstrang-Oligonukleotid wird derivatisiertem) 2-Hydroxymercaptoethanol oder ein anderer Thiol- oder Disulfid-Linker geeigneter Kettenlänge 10⁻⁵ bis 10⁻¹ molar zugesetzt (je nach gewünschtem Inter-Oligonukleotid-Abstand), das gemeinsam mit dem Einzelstrang Oligonukleotid an die Goldoberfläche koadsorbiert. (c) Herstellung einer Au-ss-oligo-PQQ Elektrode wie unter Beispiel 2 beschrieben, aber unter Weglassen des 0.7 molaren Zusatzes an Elektrolyten (im Beispiel TEATFB) im Inkubationsschritt zur Derivatisierung der Goldoberfläche mit (doppelt derivatisiertem) Einzelstrang-Oligonukleotid. Die Phosphatgruppen und Basen-Sickstoffatome des Oligonukleotids sind durch die Abwesenheit des Salzes elektrostatisch nicht abgeschirmt und wechselwirken stark mit der Goldoberfläche. Dadurch kommt es zu einer flachen Anlagerung der Oligonukleotide auf der Elektrodenoberfläche (ϕ > 60 °) und es werden pro Flächeneinheit deutlich weniger Oligonukleotide gebunden. Anschließend können die Oligonukleotide wieder in die 27

gewünschte Position gebracht werden, indem in einem 2. Inkubationsschritt (vor oder nach Anbringen des PQQ) 2-Hydroxy-mercaptoethanol oder ein anderer Thioloder Disulfid-Linker geeigneter Kettenlänge kovalent an die noch freien Oberflächen-Goldatome angebunden wird. Dazu wird die weniger dicht mit Einzelstrang-Oligonukleotid belegte Elektrode vor oder nach Modifikation mit PQQ (Au-S-ss-oligo bzw. Au-S-ss-oligo-PQQ) mit einer ca. 5x10⁻² molaren Lösung des 2-Hydroxy-mercaptoethanols oder eines andereren Thiol- oder Disulfid-Linkers geeigneter Kettenlänge in Ethanol oder HEPES Puffer (bzw. einem Gemisch daraus, abhängig von der Löslichkeit des Thiols) benetzt und 2-24h inkubiert.

Beispiel 5: Durchführung der cyclovoltammetrischen Messungen. Die cyclovoltammetrischen Messungen wurden mit einem Computer-kontrolliertem Bipotentiostaten (CH Instruments, Model 832) bei Raumtemperatur in einer Standard Zelle mit 3-Elektroden-Anordung vermessen. Die modifizierte Goldelektrode wurde als Arbeitselektrode verwendet, als Hilfselektrode (Gegenelektrode) diente ein Platindraht und als Referenzelektrode zur Potentialbestimmung wurde eine über eine Luggin Fapillare vom Probenraum abgetrennte Ag/AgCI-Elektrode mit interner gesättigter KCI Lösung verwendet. Als Elektrolyt diente 0.7 molares TEATFB oder 1 molares NaCl. Ein Cyclovaltagramm der Au-S-ds-oligo-PQQ Elektrode im Vergleich zu einer Au-S-ss-oligo-PQQ Elektrode ist in Figur 4 gezeigt, die Auswirkung der 2 Bp Mismatches auf das Cyclovoltagramm der Au-S-ds-oligo-PQQ Elektrode ist in Abbildung 5 dargestellt. Die Potentiale sind jeweils als E-E₀, also relativ zum Halbstufenpotential angegeben.

Aus Figur 4 ist ein deutlich erhöhter Stromfluß bei Vorliegen eines Doppelstrang-Oligonukleotids gegenüber der nicht hybridisierten Form zu erkennen. Dadurch können sequenzspezifische Hybridisierungsereignisse detektiert werden. Aus Figur 5 wird deutlich, daß bei Hybridisierung mit einem Target-Oligonukleotid-Strang, der 2 Basenpaar-Mismatches aufweist zum einen ein geringerer Strom fließt, zum anderen die Differenz der Strommaxima vergrößert wird.

Patentansprüche

- Durch Anbindung einer redoxaktiven Substanz modifiziertes Nukleinsäure-Oligomer, dadurch gekennzeichnet, daß die redoxaktive Substanz bei einem Potential φ selektiv oxidierbar und reduzierbar ist, wobei φ der Bedingung 2,0 V ≥ φ ≥ - 2,0 V, gemessen gegen Normalwasserstoffelektrode, genügt.
- 2. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 1, wobei als redoxaktive Substanz ein Farbstoff verwendet wird, insbesondere ein Flavin-Derivat, ein Porphyrin-Derivat, ein Chlorophyl-Derivat oder ein Bakteriochlorophyl-Derivat.
- 3. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 1, wobei als redoxaktive Substanz ein Chinon, insbesondere ein Pyrrolo-Chinolin-Chinon (PQQ), ein 1,4-Benzochinon, ein 1,2-Naphtochinon, ein 1,4-Naphtochinon oder ein 9,10-Anthrachinon verwendet wird.
- 4. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei die redoxaktive Substanz kovalent alternativ an eine der Phosphorsäure-, Carbonsäure- oder Amin-Einheiten, an eine der Zucker-Einheiten oder an eine der Basen des Nukleinsäure-Oligomers angebunden ist, insbesondere an eine enständige Einheit des Nukleinsäure-Oligomers.
- Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 3, wobei die redoxaktive Substanz kovalent an einen verzweigten oder unverzweigten Molekülteil beliebiger Zusammensetzung und Kettenlänge angebunden ist und der verzweigte oder unverzweigte Molekülteil alternativ an eine der Phosphorsäure-, Carbonsäure- oder Amin-Einheiten, an eine der Zucker-Einheiten oder an eine der Basen des Nukleinsäure-Oligomers angebunden ist, insbesondere an eine enständige Einheit des Nukleinsäure-Oligomers.
- 6. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 5, wobei die redoxaktive Substanz kovalent an einen verzweigten oder unverzweigten Molekülteil angebunden ist, dessen kürzeste durchgehende Verbindung zwischen den verbundenen Strukturen 1 bis 14 Atome umfaßt.
- 7. Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei das modifizierte Nukleinsäure-Oligomer sequenzspezifisch Einzelstrang-DNA, RNA und/oder PNA binden kann.

- 8. Modifiziertes Nukleinsäure-Oligomer nach Anspruch 7, wobei das modifizierte Nukleinsäure-Oligomer ein Desoxyribonukleinsäure-, Ribonukleinsäure-, ein Peptidnukleinsäure-Oligomer oder ein Nukleinsäure-Oligomer mit strukturell analogem Rückgrat ist.
- 9. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers gemäß den Ansprüchen 1 bis 4, dadurch gekennzeichnet, daß die redoxaktive Substanz an ein Nukleinsäure-Oligomer gebunden wird, wobei die Anbindung an eine Phosphorsäure- oder Carbonsäure-Gruppe des Nukleinsäure-Oligomers durch Amidbildung mit (primären oder sekundären) einer Aminogruppe der redoxaktiven Substanz, durch Esterbildung mit einer (primären, sekundären oder tertiären) Alkohol-Gruppe der redoxaktiven Substanz, oder durch Thioesterbildung mit einer (primären, sekundären oder tertiären) Thio-Alkohol-Gruppe der redoxaktiven Substanz bzw. durch Kondensation einer Amin-Gruppe des Nukleinsäure-Oligomers mit einer Aldehyd-Gruppe der redoxaktiven Substanz erfolgt.
- 10. Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers gemäß den Ansprüchen 5 bis 8, dadurch gekennzeichnet, daß die redoxaktive Substanz an einen verzweigten oder unverzweigten Molekülteil beliebiger Zusammensetzung und Kettenlänge kovalent angebunden wird, wobei die Anbindung an eine Phosphorsäure- oder Carbonsäure-Gruppe des verzweigten oder unverzweigten Molekülteils durch Amidbildung mit (primären oder sekundären) einer Aminogruppe der redoxaktiven Substanz, durch Esterbildung mit einer (primären, sekundären oder tertiären) Alkohol-Gruppe der redoxaktiven Substanz, oder durch Thioesterbildung mit einer (primären, sekundären oder tertiären) Thio-Alkohol-Gruppe der redoxaktiven Substanz bzw. durch Kondensation einer Amin-Gruppe des verzweigten oder unverzweigten Molekülteils mit einer Aldehyd-Gruppe der redoxaktiven Substanz erfolgt.
- 11. Modifizierte leitfähige Oberfläche, dadurch gekennzeichnet, daß eine oder mehrere Arten von modifizierten Nukleinsäure-Oligomeren gemäß den Ansprüchen 1 bis 8 an eine leitfähige Oberfläche angebunden sind.
- 12. Modifizierte leitfähige Oberfläche nach Anspruch 11, wobei die Oberfläche aus einem Metall oder einer Metallegierung besteht, insbesondere einem Metall ausgewählt aus der Gruppe Platin, Palladium, Gold, Cadmium, Quecksilber,

Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium, Mangan und deren Mischungen.

- 13. Modifizierte leitfähige Oberfläche nach Anspruch 11, wobei die Oberfläche aus einem Halbleiter besteht, insbesondere einem Halbleiter ausgewählt aus der Gruppe Kohlenstoff, Silizium, Germanium und α -Zinn.
- 14. Modifizierte leitfähige Oberfläche nach Anspruch 11, wobei die Oberfläche aus einer binären Verbindung der Elemente der Gruppen 14 und 16, einer binären Verbindung der Elemente der Gruppen 13 und 15, einer binären Verbindung der Elemente der Gruppen 15 und 16, oder einer binären Verbindung der Elemente der Gruppen 11 und 17 besteht, insbesondere aus einem Cu(I)-Halogenid oder einem Ag(I)-Halogenid.
- 15. Modifizierte leitfähige Oberfläche nach Anspruch 11, wobei die Oberfläche aus einer ternären Verbindung der Elemente der Gruppen 11, 13 und 16 oder einer ternären Verbindung Elemente der Gruppen 12, 13 und 16 besteht.
- 16. Modifizierte leitfähige Oberfläche nach den Ansprüchen 11 bis 15, wobei die modifizierten Nukleinsäure-Oligomere an die leitfähige Oberfläche kovalent oder durch Physisorption angebunden sind.
- 17. Modifizierte leitfähige Oberfläche nach Anspruch 16, wobei alternativ eine der Phosphorsäure-, Carbonsäure- oder Amin-Einheiten, eine der Zucker-Einheiten oder eine der Basen des Nukleinsäure-Oligomers kovalent oder durch Physisorption an die leitfähige Oberfläche angebunden ist, insbesondere eine enständige Einheit des Nukleinsäure-Oligomers.
- 18. Modifizierte leitfähige Oberfläche nach einem der Ansprüche 11 bis 15, wobei an die leitfähige Oberfläche verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent oder durch Physisorption angebunden sind und die modifizierten Nukleinsäure-Oligomere kovalent an diese Molekülteile angebunden sind.
- 19. Modifizierte leitfähige Oberfläche nach Anspruch 18, wobei das verzweigte oder unverzweigte Molekülteil eine kürzeste durchgehende Verbindung zwischen den verbundenen Strukturen von 1 bis 14 Atome umfaßt.

- 20. Modifizierte leitfähige Oberfläche nach den Ansprüchen 18 und 19, wobei der verzweigte oder unverzweigte Molekülteil alternativ an eine der Phosphorsäure-, Carbonsäure- oder Amin-Einheiten, an eine der Zucker-Einheiten oder an eine der Basen des Nukleinsäure-Oligomers kovalent gebunden ist, insbesondere an eine enständige Einheit des Nukleinsäure-Oligomers.
- 21. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 11 bis 20, wobei ein oder mehrere Arten von modifizierten Nukleinsäure-Oligomeren gemäß den Ansprüchen 1 bis 8 auf eine leitfähige Oberfläche aufgebracht werden.
- 22. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 11 bis 20, wobei eine oder mehrere Arten von Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche gebunden werden und ausschließlich die an die leitfähige Oberfläche gebundenen Nukleinsäure-Oligomere durch Anbindung einer redoxaktiven Substanz an die Nukleinsäure-Oligomere modifiziert werden.
- 23. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach Anspruch 22, wobei die Anbindung der redoxaktiven Substanz an das Nukleinsäure-Oligomer durch Reaktion der redoxaktiven Substanz mit einer Phosphorsäure-Einheit, einer Zucker-Einheit oder einer der Basen des Nukleinsäure-Oligomers erfolgt, insbesondere durch Reaktion mit einer enständigen Einheit des Nukleinsäure-Oligomers.
- 24. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach Anspruch 22, wobei die redoxaktive Substanz kovalent an einen verzweigten oder unverzweigten Molekülteil beliebiger Zusammensetzung und Kettenlänge angebunden wird und der verzweigte oder unverzweigte Molekülteil alternativ an eine der Phosphorsäure-, Carbonsäure- oder Amin-Einheiten, an eine der Zucker-Einheiten oder an eine der Basen des Nukleinsäure-Oligomers angebunden wird, insbesondere an eine enständige Einheit des Nukleinsäure-Oligomers.
- 25. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 21 bis 24, wobei das Nukleinsäure-Oligomer oder das modifizierte Nukleinsäure-Oligomer mit dem dazu komplementären Nukleinsäure-Oligomerstrang hybridisiert wird und in Form des Doppelstranghybrids auf die leitfähige Oberfläche aufgebracht wird.

- 26. Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 21 bis 25, wobei das Nukleinsäure-Oligomer oder das modifizierte Nukleinsäure-Oligomer in Gegenwart von weiteren chemischen Verbindungen, die ebenfalls an die leitfähige Oberfläche angebunden werden, auf die leitfähige Oberfläche aufgebracht wird.
- 27. Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybridisierungsereignissen, dadurch gekennzeichnet, daß eine leitfähige Oberfläche, wie in den Ansprüchen 11 bis 20 definiert, mit Nukleinsäure-Oligomeren in Kontakt gebracht wird mit Nukleinsäure-Oligomeren in Kontakt gebracht werden und anschließend eine Detektion der elektrischen Kommunikation zwischen der redoxaktiven Einheit und der jeweiligen leitfähigen Oberfläche erfolgt.
- 28. Verfahren nach Anspruch 27, wobei die Detektion cyclovoltametrisch, amperometrisch oder durch Leitfähigkeitsmessung erfolgt.

WO 00/31101 PCT/EP99/08888

Fig. 1a

1/4

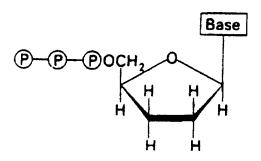


Fig. 1b

DNA-Fragment

Primer

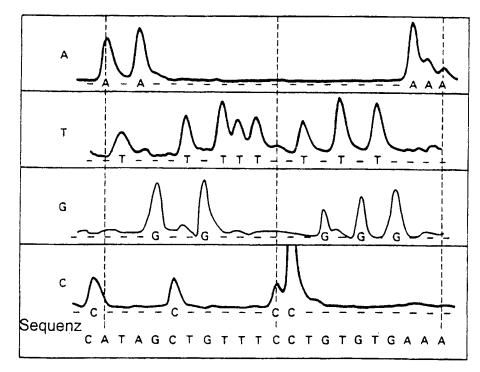
DNA-Polymerase I dATP, dTTP, dCTP, dGTP Didesoxyanalogon von dATP

3'----GAATTCGCTAATGC------5'--CTTAAGCGATTA

3'----GAATTCGCTAATGC------5'--CTTAAGCGA

Fig. 1c

Fluoreszenzintensität



Länge des Oligonukleotids

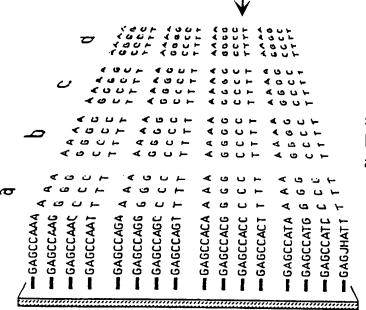
ERSATZBLATT (REGEL 26)

Fig. 2

2/4

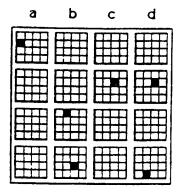
DNA-Fragment

5'-... AGTCCCTTGGCTC...-3'



spezifische Hybridisierung an Oktamermatrix

Sequanzbestimmung durch Mustererkennung



3'-TCAGGGAA-5'
3'-CAGGGAAC-5'
3'-AGGGAACC-5'
3'-GGGAACCG-5'
3'-GGAACCGA-5'
3'-GAACCGAG-5'

Oktamer 1
Oktamer 2
Oktamer 3
Oktamer 4
Oktamer 5
Oktamer 6

Ψ

3'-TCAGGGAACCGAG-5' zusammengesetzte Komplementärsequenz

 ψ

5'-...AGTCCCTTGGCTC...-3' deduzierte Sequenz des DNA-Fragments

ERSATZBLATT (REGEL 26)

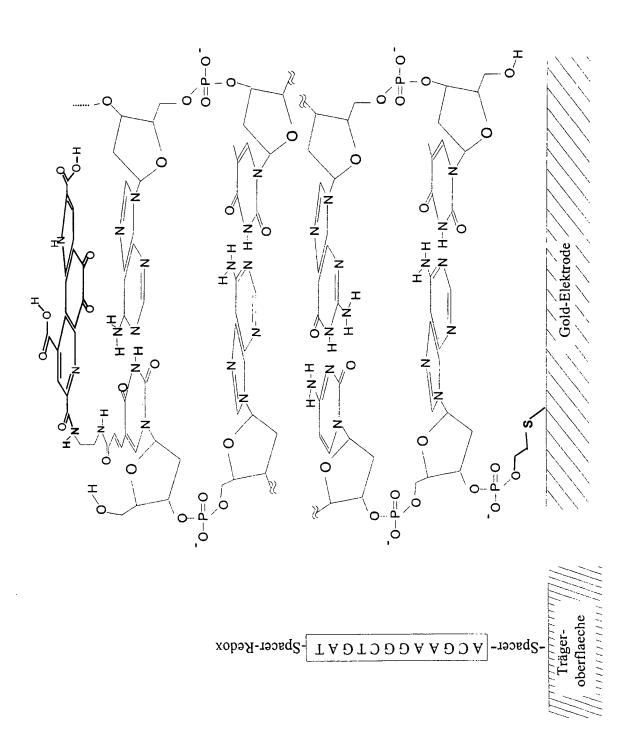


Fig. 3

4/4

Fig. 4

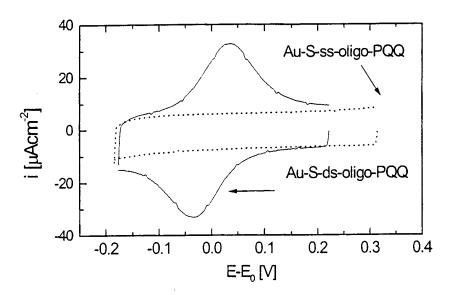
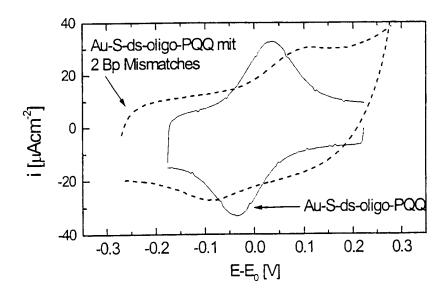


Fig. 5



INTERNAT: JAL SEARCH REPORT

In July Application No

		FC	1/67 99/08888	
A. CLASS IPC 7	GIFICATION OF SUBJECT MATTER C07H21/00 C12Q1/68			
According	to International Patent Classification (IPC) or to both national class	sification and IPC		
	SEARCHED			
IPC 7	ocumentation searched (classification system followed by classifi C07H C12Q	cation symbols)		
Documenta	ation searched other than minimum documentation to the extent th	at such documents are included i	n the fields searched	
Electronic o	data base consulted during the international search (name of data	base and, where practical, searc	h terms used)	
C. DOCUM	ENTS CONSIDERED TO BE RELEVANT			
Category '	Citation of document, with indication, where appropriate, of the	relevant passages	Relevant to claim No.	
A	Functoinalized Monolayers of Redox Proteins on Electrode Surfaces: Novel 27,28 Bioelectronic and Optobioelectronic systems." BIOSENSORS AND BIOELECTRONICS,		1,3,9, 11,21, 27,28	
	vol. 12, no. 4, 1997, pages 337 XP000874626 figures 7-10 	-356, -/		
X Funt	er documents are listed in the continuation of box C	X Patent family member	s are listed in annex.	
"A" document defining the general state of the lart which is not considered to be of particular relevance." "E" earlier document but published on or after the international filling date. "L" document which may throw doubts on pnority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified). "O" document referring to an oral disclosure, use, exhibition or other means. "P" document published prior to the international filling date but later than the pnority date claimed.		To later document published after the international filling date or priority date and not in conflict with the application but cifed to understand the principle or theory underlying the invention. X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone. Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.		
	Schoulance 2000	Date of mailing of the interior	national search report	
	February 2000	01/03/2000 Authorized officer		
	European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Scott, J		

Form PCT/ISA/210 (second sheet) (July 1992)

INTI ATIONAL SEARCH REPORT

Ir ational Application No PCT/EP 99/08888

Cicontinuation) DOCUMENTS CONSIDERED TO BE RELEVANT Category Citation of document, with indication, where appropriate, of the relevant passages A MILLAN K M ET AL: "VOLTAMMETRIC DNA BIOSENSOR FOR CYSTIC FIBROSIS BASED ON A MODIFIED CARBON PASTE ELECTRODE" ANALYTICAL CHEMISTRY, US, AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 66, no. 18, 15 September 1994 (1994–09–15), pages 2943–2948, XP000478033 ISSN: 0003–2700 abstract A JOHNSTON D H ET AL: "ELECTROCHEMICAL MEASUREMENT OF THE SOLVENT ACCESSIBILITY OF NUCLEOBASES USING ELECTRON TRANSFER BETWEEN DNA AND METAL COMPLEXES" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, US, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC. vol. 117, no. 35, 1995, pages 8933–8938–8938, XP002900562 ISSN: 0002–7863 A N.NAKAMURA ET AL.: "Electrochemical Properties of Coenzyme Pyrroloquinolinequinone Using a Di-(4-pyridyl) Disulfide Modified Gold Electrode under Acidic Conditions." BULLETIN O FTHE CHEMICAL SOCIETY OF JAPAN, vol. 66, April 1993 (1993–04), pages 1289–1291, XP000867849 abstract A EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25 March 1998 (1998–03–25)	1,3,9, 11,21, 27,28
BIOSENSOR FOR CYSTIC FIBROSIS BASED ON A MODIFIED CARBON PASTE ELECTRODE" ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 66, no. 18, 15 September 1994 (1994-09-15), pages 2943-2948, XP000478033 ISSN: 0003-2700 abstract A JOHNSTON D H ET AL: "ELECTROCHEMICAL MEASUREMENT OF THE SOLVENT ACCESSIBILITY OF NUCLEOBASES USING ELECTRON TRANSFER BETWEEN DNA AND METAL COMPLEXES" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY,US,AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC. vol. 117, no. 35, 1995, pages 8933-8938-8938, XP002900562 ISSN: 0002-7863 A N.NAKAMURA ET AL.: "Electrochemical Properties of Coenzyme Pyrroloquinolinequinone Using a Di-(4-pyridyl) Disulfide Modified Gold Electrode under Acidic Conditions." BULLETIN O FTHE CHEMICAL SOCIETY OF JAPAN, vol. 66, April 1993 (1993-04), pages 1289-1291, XP000867849 abstract A EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25 March 1998 (1998-03-25)	11,21, 27,28
MEASUREMENT OF THE SOLVENT ACCESSIBILITY OF NUCLEOBASES USING ELECTRON TRANSFER BETWEEN DNA AND METAL COMPLEXES" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, US, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, vol. 117, no. 35, 1995, pages 8933-8938-8938, XP002900562 ISSN: 0002-7863 A N.NAKAMURA ET AL.: "Electrochemical Properties of Coenzyme Pyrroloquinolinequinone Using a Di-(4-pyridyl) Disulfide Modified Gold Electrode under Acidic Conditions." BULLETIN O FTHE CHEMICAL SOCIETY OF JAPAN, vol. 66, April 1993 (1993-04), pages 1289-1291, XP000867849 abstract A EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25 March 1998 (1998-03-25)	11,21,27,28
Properties of Coenzyme Pyrroloquinolinequinone Using a Di-(4-pyridyl) Disulfide Modified Gold Electrode under Acidic Conditions." BULLETIN O FTHE CHEMICAL SOCIETY OF JAPAN, vol. 66, April 1993 (1993-04), pages 1289-1291, XP000867849 abstract A EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25 March 1998 (1998-03-25)	
25 March 1998 (1998-03-25)	11,21,27,28
claims 1-29	1,3,9, 11,21, 27,28
A WO 96 40712 A (CALIFORNIA INST OF TECHN) 19 December 1996 (1996-12-19) claims 1-27; example 8	1,3,9, 11,21, 27,28
A US 5 312 527 A (MIKKELSEN SUSAN R ET AL) 17 May 1994 (1994-05-17) abstract	1,3,9, 11,21, 27,28
WO 95 15971 A (CALIFORNIA INST OF TECHN) 15 June 1995 (1995-06-15) the whole document	1.3,9, 11,21, 27,28

INTERNATIO ... L SEARCH REPORT

in itio. optication No PCT/EP 99/08888

	C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT						
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.					
A	KATZ E ET AL: "ELECTROCHEMICAL STUDY OF PYRROLOQUINOLINE QUINONE COVALENTLY IMMOBILIZED AS A MONOLAYER ONTO A CYSTAMINE-MODIFIED GOLD ELECTRODE" JOURNAL OF ELECTROANALYTICAL CHEMISTRY AND INTERFACIAL ELECTROCHEMISTRY, NL, ELSEVIER, AMSTERDAM, vol. 367, 1 January 1994 (1994-01-01), pages 59-70, XP000672483 ISSN: 0022-0728 the whole document	1,3,9, 11,21, 27,28					
P,A	WO 99 51778 A (BARTON JACQUELINE ;HILL MICHAEL (US); KELLEY SHANA (US); CALIFORNI) 14 October 1999 (1999-10-14) examples 1-15	1,3,9, 11,21, 27,28					

INTERNATIONAL SEARCH REPORT

International application No. PCT/EP 99/08888

Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) Box 1 This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons: Claims Nos. 2, 13 because they relate to subject matter not required to be searched by this Authority, namely: See supplemental sheet ADDITIONAL MATTER PCT/ISA/210 Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically: Claims Nos: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a) Observations where unity of invention is lacking (Continuation of item 2 of first sheet) This International Searching Authority found multiple inventions in this international application, as follows: As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos... No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims, it is covered by claims Nos.: The additional search fees were accompanied by the applicant's protest. Remark on Protest No protest accompanied the payment of additional search fees

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/08888

ADDITIONAL MATTER PCT/ISA/210

Field I.2 (continued)

Claims nos.:

2, 13

The valid patent claims relates to a disproportionally large number of possible compounds of which only a small portion can be supported by the description under the terms of Article 6 of the PCT or can be considered disclosed in the patent application under the terms of Article 5 of the PCT. In the case in question, the patent claims lack the corresponding support and the patent application lacks the corresponding disclosure to such a degree that a meaningful search with respect to the entire scope of protection sought seems impossible. As a result the search was directed towards those parts of the patent claims which appear supported and disclosed in the above sense, i.e. the parts relating to compounds containing pyrrolo-quinoline quinone as redox-active substance and compounds containing pyrrolo-quinoline quinone as the redox-active substance together with a gold electrode.

In addition, the valid patent claim no. I relates to products which are characterized by a desirable peculiarity or property, i.e. compounds defined by redox activity in a potential range of between -2.0V and 2.0V.

The patent claims therefore relate to all products presenting the above peculiarity or property, whereas the patent claim by the description under the terms of Article 6 of the PCT provides support for only a limited number of such products. In the case in question, the patent claims lack the corresponding support and the patent application lacks the corresponding disclosure to such a degree that a meaningful search with respect to the entire scope of protection sought seems impossible. Moreover, the patent claims also lack the clarity required under the terms of Article 6 of the PCT since an attempt is made in said patent claims to define the compounds in terms of their desired results (see above).

The applicant is reminded that claims relating to inventions in respect of which no international search report has been established cannot normally be the subject of an international preliminary examination (Rule 66.1 (e) PCT). As a general rule, the EPO in its capacity as the authority entrusted with the task of carrying out an international preliminary examination will not conduct a preliminary examination for subjects in respect of which no search has been provided. This also applies to cases where the patent claims were amended after receipt of the international search report (Article 19 PCT) or to cases where the applicant provides new patent claims in keeping with the procedure mentioned in Chapter II of the PCT.

INTEF TIONAL SEARCH REPORT

Information on patent family members

int Itional Application No PCT/EP 99/08888

			,				, 00000
	atent document d in search repor	t	Publication date		Patent family member(s)		Publication date
ΕP	0831327	Α	25-03-1998	DE	19639169) A	02-04-1998
				CA	2216109		24-03-1998
				JP	10130247		19-05-1998
WO	9640712	Α	19-12-1996	US	5824473	 B A	20-10-1998
				ΑU	6166296		30-12-1996
				EP	0871642	? A	21-10-1998
				US	5770369) A	23-06-1998
				US	5952172	. A	14-09-1999
US	5312527	Α	17-05-1994	NONE			*
WO	9515971	Α	15-06-1995	US	5591578		07-01-1997
				ΑU	703329		25-03-1999
				ΑŲ	1215295		27-06-1995
				CA	2178618	Α	15-06-1995
				EP	0733058	Α	25-09-1996
				JP	9506510		30-06-1997
				US	5824473		20-10-1998
				US	5770369		23-06-1998
				UŞ	5780234		14-07-1998
				US	5705348		06-01-1998
				US	5952172	Α	14-09-1999

Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONALERCHERCHENBERICHT

PCT/EP 99/08888

IPK 7	SIFIZIERUNG DES ANMELDUNGSGEGENSTANDES C07H21/00 C12Q1/68			
Nach der i	internationalen Patentklassifikation (IPK) oder nach der nationalen K	(lassifikation und der IPK		
	ERCHIERTE GEBIETE			
Recherchii IPK 7	erter Mindestprufstoff (Klassifikationssystem und Klassifikationssym C07H C12Q	nbole)		
Recherchie	erte aber nicht zum Mindestprufstoff genorende Veröffentlichungen.	soweit diese unter die recherchierten Gebiete	e fallen	
Wahrend d	fer internationalen Recherche konsultierte elektronische Datenbank	(Name der Datenbank und evtl. verwendete	Suchbegntfe)	
C. ALS W	ESENTLICH ANGESEHENE UNTERLAGEN			
Kategorie,	Bezeichnung der Veröffentlichung, soweit erforderlich unter Anga	ibe der in Betracht kommenden Teile	Betr. Anspruch Nr.	
Α	I.WILLNER ET AL.: "Assembly of Functoinalized Monolayers of Red Proteins on Electrode Surfaces: Bioelectronic and Optobioelectro systems." BIOSENSORS AND BIOELECTRONICS, Bd. 12, Nr. 4, 1997, Seiten 337-XP000874626 Abbildungen 7-10	Novel nic	1,3,9, 11,21, 27,28	
		-/		
	tere Veroffentlichungen sind der Fortsetzung von Feld C zu lehmen	X Siehe Anhang Patentfamilie		
 Besondere Kategorien von angegebenen Veröffentlichungen "A" Veröffentlichung, die den aligemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusenen ist "E" alleres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist "L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbenicht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt) "O" Veröffentlichung, die sich auf eine mundliche Öffenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht "P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beansprüchten Prioritätsdatum veröffentlich worden ist 		T" Spatere Veröffentlichung, die nach dem internationalen Anmeldedatu oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verstandnis des der Erlindung zugrundeliegenden Prinzips oder der ihr zugrundeliegende. Theorie angegeben ist "X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erlind kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erlindenscher Tätigkeit beruhend betrachtet werden "Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erlind kann nicht als auf erlindenscher Tätigkeit berühend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist.		
	Abschlusses der internationalen Recherche . Februar 2000	Absendedatum des internationalen Reci	herchenberichts	
Name und F	Postanschrift der Internationalen Recherchenbehorde Europaisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.	Bevollmächtigter Bediensteter		
	Fax: (+31-70) 340-3016	Scott, J	i	

INTERNATION OR RECHERCHENBERICHT

li ationales Aktenzeichen PCT/EP 99/08888

1,3,9, 11,21, 27,28
11,21,
1,3,9, 11,21, 27,28

INTERNATIONALER RE .. IERCHENBERICHT

Ir at. s Aktenzeichen
PCT/EP 99/08888

	rung) ALS WESENTLICH ANGESEHENE UNTERLAGEN		
Kategorie -	Bezeichnung der Veröffentlichung, soweit erfordersch unter Angabe der in Betracht komm	nenden Teile	Betr. Anspruch Nr.
A	KATZ E ET AL: "ELECTROCHEMICAL STUDY OF PYRROLOQUINOLINE QUINONE COVALENTLY IMMOBILIZED AS A MONOLAYER ONTO A CYSTAMINE-MODIFIED GOLD ELECTRODE" JOURNAL OF ELECTROANALYTICAL CHEMISTRY AND INTERFACIAL ELECTROCHEMISTRY, NL, ELSEVIER, AMSTERDAM, Bd. 367, 1. Januar 1994 (1994-01-01), Seiten 59-70, XP000672483 ISSN: 0022-0728 das ganze Dokument		1,3,9, 11,21, 27,28
Ρ,Α	das ganze Dokument WO 99 51778 A (BARTON JACQUELINE ;HILL MICHAEL (US); KELLEY SHANA (US); CALIFORNI) 14. Oktober 1999 (1999-10-14) Beispiele 1-15		1,3,9, 11,21, 27,28

INTERNATIONALER RECHERCHENBERICHT

internationales Aktenzeichen

PCT/EP 99/08888

Feld I Bemerkungen zu den Anspruchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 2 auf Blatt
Gemaß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:
Ansprüche Nr. weil sie sich auf Gegenstande beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, namfich
2. X Anspruche Nr. 2.13 weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, namlich siehe Zusatzblatt WEITERE ANGABEN PCT/ISA/210
3. Anspruche Nr. weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.
Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 3 auf Blatt 1)
Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthalt:
Da der Anmelder alle erforderlichen zusätzlichen Recherchengebuhren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche.
Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden könnte, der eine zusatzliche Recherchengebuhr gerechtfertigt hatte, hat die Behorde nicht zur Zahlung einer solchen Gebuhr aufgefordert.
Da der Anmelder nur einige der erforderlichen zusatzlichen Recnerchengebuhren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Anspruche, für die Gebuhren entrichtet worden sind, nämlich auf die Anspruche Nr. Anspruche Nr.
Der Anmeider hat die Giforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschrankt sich daher auf die in den Ansprüchen zuerst erwahnte Erfindung: diese ist in folgenden Ansprüchen erfaßt:
Bemerkungen hinsichtlich eines Widerspruchs Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt. Die Zahlung zusätzlicher Recherchengebühren lerfolgte ohne Widerspruch.

Formblatt PCT/ISA/210 (Fortsetzung von Blatt 1 (1))(Juli 1998)

WEITERE ANGABEN

PCT/ISA/ 210

Fortsetzung von Feld I.2

Ansprüche Nr.: 2,13

Die geltenden Patentansprüche beziehen sich auf eine unverhältnismäßig große Zahl möglicher Verbindungen, von denen sich nur ein kleiner Anteil im Sinne von Art. 6 PCT auf die Beschreibung stützen und als im Sinne von Art.5 PCT in der Patentanmeldung offenbart gelten kann. Im vorliegenden Fall fehlt den Patentansprüchen die entsprechende Stütze und fehlt der Patentanmeldung die nötige Offenbarung in einem solchen Maße, daß eine sinnvolle Recherche über den gesamten erstrebten Schutzbereich unmöglich erscheint. Daher wurde die Recherche auf die Teile der Patentansprüche gerichtet, welche im o.a. Sinne als gestützt und offenbart erscheinen, nämlich die Teile betreffend Verbindungen mit Pyrrolo-Chinolino-Chinon als redoxaktive Substanz und Verbindungen mit Pyrrolo-Chinolino-Chinon als die redoxaktive Substanz zusammen mit einer Gold-Elektrode.

Desweiteren bezieht sich der geltende Patentanspruch 1 auf Produkte, die charakterisiert werden durch eine erstrebenswerte Eigenheit oder Eigenschaft, nämlich Verbindungen definiert durch Redoxaktivität in einem Potentialbereich von -2.0V bis 2.0V.

Die Patentansprüche umfassen daher alle Produkte, die diese Eigenheit oder Eigenschaft aufweisen, wohingegen die Patentanmeldung Stütze durch die Beschreibung im Sinne von Art. 5 PCT nur für eine begrenzte Zahl solcher Produkte liefert. Im vorliegenden Fall fehlt den Patentansprüchen die entsprechende Stütze bzw. der Patentanmeldung die nötige Offenbarung in einem solchen Maße, daß eine sinnvolle Recherche über den gesamten erstrebten Schutzbereich unmöglich erscheint. Desungeachtet fehlt den Patentansprüchen auch die in Art. 6 PCT geforderte Klarheit, nachdem in ihnen versucht wird, die Verbindung über das jeweils erstrebte Ergebnis zu definieren (siehe oben).

Der Anmelder wird darauf hingewiesen, daß Patentansprüche, oder Teile von Patentansprüchen, auf Erfindungen, für die kein internationaler Recherchenbericht erstellt wurde, normalerweise nicht Gegenstand einer internationalen vorläufigen Prüfung sein können (Regel 66.1(e) PCT). In seiner Eigenschaft als mit der internationalen vorläufigen Prüfung beauftragte Behörde wird das EPA also in der Regel keine vorläufige Prüfung für Gegenstände durchführen, zu denen keine Recherche vorliegt. Dies gilt auch für den Fall, daß die Patentansprüche nach Erhalt des internationalen Recherchenberichtes geändert wurden (Art. 19 PCT), oder für den Fall, daß der Anmelder im Zuge des Verfahrens gemäß Kapitel II PCT neue Patentanprüche vorlegt.

INTERNATION OR RECHERCHENBERICHT

Angapen zu Veröffentlich, "gen, die zur seiben Patentfamilie genoren

n tionales Aktenzeichen
PCT/EP 99/08888

	Recherchenberio artes Patentdoku		Datum der Veröffentlichung		litglied(er) der Patentlamilie	Datum der Veröffentlichung
EP	0831327	Α	25-03-1998	DE	19639169 A	02-04-1998
				CA	2216105 A	24-03-1998
				JP	10130247 A	19-05-1998
WO	9640712	Α	19-12-1996	US	5824473 A	20-10-1998
				AU	6166296 A	30-12-1996
				EΡ	0871642 A	21-10-1998
				US	5770369 A	23-06-1998
				US	5952172 A	14-09-1999
US	5312527	А	17-05-1994	KEIN	 IE	
MO	9515971	Α	15-06-1995	US	5591578 A	07-01-1997
				AU	703329 B	25-03-1999
				ΑU	1215295 A	27-06-1995
				CA	2178618 A	15-06-1995
				EΡ	0733058 A	25-09-1996
				JP	9506510 T	30-06-1997
				US	5824473 A	20-10-1998
				US	5770369 A	23-06-1998
				US	5780234 A	14-07-1998
				US	5705348 A	06-01-1998
				US	5952172 A	14-09-1999
WO	9951778	Α	14-10-1999	AU	3550699 A	25-10-1999

Formblatt PCT/ISA/210 (Anhang Patentlamilie)(Juli 1992)

VERTRA

BER DIE INTERNATIONALE ZU MMENARBEIT AUF DEM GEBIET DES PATENTWESENS

PCT

INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts	WEITERES siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5					
Internationales Aktenzeichen PCT/EP 00/00084	Internationales Anmelo (Tag/Monat/Jahr)	ledatum	(Frühestes) Prioritätsdatum (Tag/Monat/Jahr)			
PCT/EP 00/ 00084 07/01/2000 18/01/1999 Anmelder						
HARTWICH, Gerhard						
Dieser internationale Recherchenbericht wurd Artikel 18 übermittelt. Eine Kople wird dem Int Dieser Internationale Recherchenbericht umfa	emadonalen buro uperm	Recherchenbehörde er ittelt. Blätter.	stellt und wird dem Anmelder gemäß			
			Unterlagen zum Stand der Technik bei.			
1. Grundlage des Berichts						
 a. Hinsichtlich der Sprache ist die inter durchgeführt worden, in der sie eing 	nationale Recherche auf ereicht wurde, sofern unt	der Grundlage der interi er diesem Punkt nichts a	nationalen Anmeldung in der Sprache underes angegeben ist.			
Die internationale Recherch Anmeldung (Regel 23.1 b)) o	e ist auf der Grundlage ei Jurchgeführt worden,	ner bei der Behörde eing	gereichten Übersetzung der internationalen			
 Hinsichtlich der in der internationaler Recherche auf der Grundlage des S 	n Anmeldung offenbarten equenzprotokolls durchge	Nucleotid- und/oder A	minosäuresequenz ist die internationale			
in der internationalen Anmel	dung in Schriflicher Form	enthalten ist.				
zusammen mit der internatio			ereicht worden ist.			
	bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.					
internationalen Anmeldung ir	n Antheidezeitpunkt ninat	isgent, wurde vorgelegt.				
Die Erklärung, daß die in con wurde vorgelegt.	nputerlesbarer Form erfal	Sten Informationen dem	schriftlichen Sequenzprotokoll entsprechen,			
2. Bestimmte Ansprüche hab			e Feld I).			
3. Mangeinde Einheitlichkeit d	der Erfindung (siehe Feld	i II).				
4. Hinsichtlich der Bezeichnung der Erfind	ung					
Wird der vom Anmelder einge	reichte Wortlaut genehmi	gt.				
wurde der Wortlaut von der B	ehörde wie folgt festgese	t zt:				
5. Hinsichtlich der Zusammenfassung						
Recherchenberichts eine Stel	el 38.2b) in der in Feld III nnerhalb eines Monats na lungnahme vorlegen.	angegebenen Fassung ich dem Datum der Abso	von der Behörde festgesetzt. Der endung dieses internationalen			
6. Folgende Abbildung der Zelchnungen ist	mit der Zusammenfassur	ng zu veröffentlichen: Ab	bb. Nr. <u>6</u>			
wie vom Anmelder vorgeschla	•		keine der Abb.			
weil der Anmelder selbst keine						
weil diese Abbildung die Erfind	iung besser kennzeichne	l.	,			

INTERNATIONALE' ECHERCHENBERICHT

nationales Aktenzeichen CT/EP 00/00084

a. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES IPK 7 C12Q1/68 C07H21/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprůfstoff (Klassifikationssystem und Klassifikationssymbole) $IPK \ 7 \ C12Q \ C07H$

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultlerte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. ALS WE	C. ALS WESENTLICH ANGESEHENE UNTERLAGEN					
Kategorie°	Bezelchnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Telle	Betr. Anspruch Nr.				
X	US 5 770 369 A (FRASER SCOTT E ET AL) 23. Juni 1998 (1998-06-23) das ganze Dokument	1-55				
X	WO 98 20162 A (GOZIN MICHAEL ;YU CHANGJUN (US); KAYYEM JON F (US); CLINICAL MICRO) 14. Mai 1998 (1998-05-14) das ganze Dokument	1-5, 21-23, 29,30				
X	US 5 622 946 A (HARRIMAN ANTHONY M ET AL) 22. April 1997 (1997-04-22) Beispiel 4	1-5, 21-23				
X	DE 42 16 696 A (DEUTSCHE AEROSPACE) 28. Oktober 1993 (1993-10-28) das ganze Dokument/	1-5				

X	Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen	X Siehe Anhang Patentfamilie		
"A" V "E" ā "L" V "O" V	sondere Kategorien von angegebenen Veröffentlichungen : Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist äfteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist /eröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft er- scheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt) Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht /eröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist	adiadadashar Thristian is to a little and a		
	n des Abschlusses der internationalen Recherche 6. September 2000	Absendedatum des internationalen Recherchenberichts 13/09/2000		
Name	e und Postanschrift der Internationalen Recherchenbehörde Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340–2040, Tx. 31 651 epo nl, Fax: (+31-70) 340–3016	Bevollmächtigter Bediensteter Reuter, U		

2

ATENT COOPERATION TRUSTY

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

HARTWICH, Gerhard Nibelungenstrasse 10 D-80639 München ALLEMAGNE

Date of mailing (day/month/year) 20 July 2000 (20.07.00)			
Applicant's or agent's file reference	1100	II	MPORTANT NOTICE
International application No. PCT/EP00/00084		l date (day/month/year) 2000 (07.01.00)	Priority date (day/month/year) 18 January 1999 (18.01.99)
Applicant HARTWICH, Gerhard			

 Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice: AU,CN,JP,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

- 2. The following designated Offices have waived the requirement for such a communication at this time:
 - AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DK,EA,EE,EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW

RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

 Enclosed with this Notice is a copy of the international application as published by the International Bureau on 20 July 2000 (20.07.00) under No. WO 00/42217

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

INTERNATIONALER CHERCHENBERICHT

Angaben zu Veröffentlichunger. . . zur selben Patentfamilie gehören

mationales Aktenzeichen

| CT/EP 00/00084

	Recherchenber ührtes Patentdok		Datum der Veröffentlichung		Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
U	S 5770369	Α	23-06-1998	US	5824473 A	Veröffentlichung 20-10-1998
				ÜŚ	5591578 A	07-01-1998
				US	6071699 A	06-06-2000
				US	5952172 A	14-09-1999
				ΑU	6166296 A	30-12-1996
				EP	0871642 A	21-10-1998
				WO	9640712 A	19-12-1996
				AU	703329 B	25-03-1999
				AU	1215295 A	27-06-1995
				CA	2178618 A	15-06-1995
				EP	0733058 A	25-09-1996
				JP	9506510 T	30-06-1997
				WO	9515971 A	15-06-1995
				US	5780234 A	14-07-1998
				US	5705348 A	06-01-1998
				US	6087100 A	11-07-2000
WO	9820162	Α	14-05-1998	US	6096273 A	01-08-2000
				US	6090933 A	18-07-2000
				AU	5196798 A	29-05-1998
				EP-	0939762 A	08-09-1999
US	5622946	Α	22-04-1997	US	5457183 A	10-10-1995
				US	6072038 A	06-06-2000
				US	5583220 A	10-12-1996
				US	5580543 A	03-12-1996
				US	5587371 A	24-12-1996
				US	5632970 A	27-05-1997
				US	5801229 A	01-09-1998
				US	5969111 A	19-10-1999
				US	5888997 A	30-03-1999
				US Au	6069140 A	30-05-2000
				AU	683316 B 8075694 A	06-11-1997
				CA	2173319 A	04-05-1995
				EP	0724457 A	20-04-1995 07-08-1996
				ĴΡ	9508616 T	02-09-1997
				NO	961436 A	11-06-1996
				SG	48392 A	17-04-1998
				WO	9510307 A	20-04-1995
				ÜS	5599923 A	04-02-1997
DE	4216696	A	28-10-1993	WO	9321530 A	28-10-1993
US	5312527	A	17-05-1994	KEIN	E	
WO	9831839	Α	23-07-1998	AU	5926598 A	07-08-1998
				EP	0981643 A	01-03-2000
ΕP	0831327	Α	25-03-1998	DE	19639169 A	02-04-1998
				CA	2216105 A	24-03-1998
				JP	10130247 A	19-05-1998
				US	6057120 A	02-05-2000
WO	9951778 	Α	14-10-1999 	AU	3550699 A	25-10-1999
	0031101	Α	02-06-2000	DE	19921940 A	15-06-2000

INTERNATIONALE TCHERCHENBERICHT

nationales Aktenzeichen

· C/Fortnets	ung) ALS WESENTLICH ANGESEHENE UNTERLAGEN	00/00084
Categorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
	S S S S S S S S S S S S S S S S S S S	Dell, Anspiden Mr.
4	US 5 312 527 A (MIKKELSEN SUSAN R ET AL) 17. Mai 1994 (1994-05-17) das ganze Dokument	1-55
	KATZ E ET AL: "ELECTROCHEMICAL STUDY OF PYRROLOQUINOLINE QUINONE COVALENTLY IMMOBILIZED AS A MONOLAYER ONTO A CYSTAMINE-MODIFIED GOLD ELECTRODE" JOURNAL OF ELECTROANALYTICAL CHEMISTRY AND INTERFACIAL ELECTROCHEMISTRY, NL, ELSEVIER, AMSTERDAM, Bd. 367, 1994, Seiten 59-70, XP000672483 ISSN: 0022-0728 das ganze Dokument	1-55
	WO 98 31839 A (HARVARD COLLEGE) 23. Juli 1998 (1998-07-23) das ganze Dokument	1-55
	EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25. März 1998 (1998-03-25) das ganze Dokument	1-55
,х	WO 99 51778 A (BARTON JACQUELINE ;HILL MICHAEL (US); KELLEY SHANA (US); CALIFORNI) 14. Oktober 1999 (1999-10-14) das ganze Dokument	1-5, 21-23, 29,30
	WO 00 31101 A (HARTWICH GERHARD; HELLER ADAM (US)) 2. Juni 2000 (2000-06-02) das ganze Dokument	1-55

PCT

ANTRAG

Der Unterzeichnete beantragt, daß die vorliegen de internationale Anmeldung nach dem Vertragüber die internationale Zusammen arbeitauf dem Gebiet des Patentwesensbehandelt wird.

VomAnmeldeamtauszufüllen

PCT/EP 0 0 / 0 0 0 8 4

07 JAN 2000

InternationalesAnmeldedatum **EUROPEAN PATENT OFFICE** PCT INTERNATIONAL APPLICATION

Namedes An meldeamts und "PCT International Application"

Aktenzeichendes Anmeldersoder Anwalts (falls gewünscht)

	(max. 12 Zeichen)
FeldNr.I BEZEICHNUNGDERERFINDUNG	
Verfahren zur elektrochemischen Detektion von	Nukleinsäure-Oligomer-Hybridisierungsereignissen
FeldNr.II ANMELDER	
Name und Anschrift: (Familienname, Vorname; bei juristische Bezeichnung. Bei der Anschrift sind die Postleitzahl und der N in diesem Feld in der Anschrift angegebene Staat ist der Staat Anmelders, sofern nachstehend kein Staat des Sitzes oder Woh	n Personen vollständige amtliche lame des Staats anzugeben. Der t des Sitzes oder Wohnsitzes des insitzes angegeben ist.) DiesePersonist gleichzeitigErfinder
HARTWICH, Gerhard Nibelungenstraße 10 D-80639 München	Telefonnr.: (089) 13939899
Deutschland	Telefaxnr.: (089) 13937450
	Fernschreibnr.:
Staatsangehörigkeit(Staat): DE	SitzoderWohnsitz(Staat): DE
DiesePersonistAnmelder fürfolgendeStaaten: alleBestimmungsstaaten	ungsstaatermitAusnahme nurdieVereinigten dicimZusatzfeld senStaatenvonAmerika StaatenvonAmerika dicimZusatzfeld angegebener6taaten
FeldNr.III WEITEREANMELDERUND/ODER(W	
Name und Anschrist: (Familienname, Vorname; bei juristischei Bezeichnung, Bei der Anschrist sind die Postleitzahl und der N in diesem Feld in der Anschrist angegebene Staat ist der Staat Anmelders, sosern nachstehend kein Staat des Sitzes oder Woh	n Personen voltstandige amtliche lame des Staats anzugeben. Der des Sitzes oder Wohnsitzes des insitzes angegeben ist.) DicscPcrsonist: nurAnmcldcr AnmcldcrundErfindcr nurErfinder (Wird dieses Kästcher angekreuzt, so sind die nachstehenden Angaben nicht nötig.)
Staatsangehörigkeit(Staat):	SitzoderWohnsitz(Staat):
DiesePersonistAnmelder alleBestimmüngsstaaten alleBestimmungsstaaten derVereinigte	ungsstaatermitAusnahme nurdieVereinigten dieimZusatzfeld enStaatenvonAmerika StaatenvonAmerika dieimZusatzfeld
WeitereAnmelderund/oder(weitere)Erfindersindaufei	nemFortsetzungsblattangegeben.
FeldNr.IV ANWALTODERGEMEINSAMERVERT	RETER; ZUSTELLANSCHRIFT
DiefolgendePersonwirdhiermitbestellt/istbestelltworden,umf vordenzuständigeninternationalenBehördeninfolgenderEige	ürder(die)Anmelder Anwalt gemeinsamer enschaftzuhandelnals: Anwalt Vertreter
NamcundAnschrift: (Familienname, Vorname; bei juristischen Person Bei der Anschrift sind die Postleitzahl und der N HARTWICH, Gerbard Nibelungenstraße 10 D-80639 München Deutschland	en vollständige amtliche Bezeichnung. Name des Staats anzugeben.) Telefonnr.: (089) 13939899 Telefaxnr.: Fernschreibnr.:
Zustellanschrift:DiesesKästchenistanzukreuzen,wenn imobigenFeldeinespezielleZustellanschriftangegebeni	nkeinAnwaltodergemeinsamerVertreterbestelltistundstattdessen ist.

Feld	Nr.V	BESTIMMUNGVONSTAATEN							
Die fo	lgender	Bestimmungen nach Regel 4.9 Absatz a werden hiermit vorge	enomn	nen (bitte	die entsprechenden Kästchen ankreuzen; wenigstens ein Kästchen				
, ,	•	izi werden): Patent		, , , , , , , , , , , , , , , , , , , ,					
X	AP	ARITO-Patent: GH Ghana, GM Gambia, KE SZSwasiland, UG Uganda, ZW Simbabweundjeder			TZ Vereinigte Republik Tansania Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, der Vertragsstaattes Harare, Protokollern der POTT-te				
X	EA	SZSwasiland, UG Uganda, ZW SimbabweundjederweitereStaat,derVertragsstaatdesHarare-ProtokollsunddesPCTist EurasischesPatent: AM Armenien, AZ Aserbaidschan, BYBelarus, KG Kirgisistan, KZ Kasachstan, MD Republik Moldau, RURussischeFöderation, TJTadschikistan, TMTurkmenistanundjederweitereStaat,derVertragsstaatdes EurasischenPatentübereinkommensunddesPCTist							
X	ЕP	Europäisches Patent: AT Österreich, BE B DEDeutschland, DKDänemark, ESSpanien, FIFin IE Irland JT Italien, LULuxemburg MCMonaco	NI.	Jiederle	und LI Schweiz und Liechtenstein, CY Zypern, ankreich, GB Vereinigtes Königreich, GRGriechenland, ande, PT Portugal, SESchwedenundjederweitere Staat,				
X	OA	OAPI-Patent: BF Burkina Faso, BJ Benin, C CM Kamerun, GA Gabun, GN Guinea, GW Gui TDTschad, TGTogound iederweitere Staat der Ver	OAPI-Patent: BF Burkina Faso, BJ Benin, CF Zentralafrikanische Republik, CG Kongo, CI Côte d'Ivoire, CM Kamerun, GA Gabun, GNGuinea, GWGuinea-Bissau, ML Mali, MR Mauretanien, NE Niger, SN Senegal, TDTschad, TGTogound iederweiterestaat der Vertragsstaat der Population (NE Niger, SN Senegal,						
Natio	nales	oder ein sonstiges Verfahren gewünscht wird, bitte auf der ge Patent (falls eine andere Schutzrechtsart oder ein sonstiges	punkte Varial	eien Linie	e angenen)				
X	AE	VereinigteArabischeEmirate	v erjai		unschi wira, bitte auf der gepunkteten Linie angeben): Liberia				
X		Albanien	X						
X		Armenien			Lesotho				
X		Österreich	X		Litauen				
X		Australien	X		Luxemburg				
X		Aserbaidschan	X		Lettland				
X		Bosnien-Herzegowina	X		Republik Moldau				
[X]			X	MG	Madagaskar				
<u> </u>		Barbados	X	MK	DieehemaligejugoslawischeRepublik				
		Bulgarien	_		Mazedonien				
X		Brasilien	X		Mongolei				
X		Belarus	X	MW	/ Malawi				
X		Kanada	X	MX	Mexiko				
×		ndLI SchweizundLiechtenstein	X		Norwegen				
X	CN	China	X	NZ	Neuseeland				
X		Kuba D. CR Costa Rica	X		Polen				
Z	CZ	Tschechische Republik	X		Portugal				
		Deutschland	X		Rumänien				
X	DK	Dänemark DM Dominika	X	RU	RussischeFöderation				
X	EE	Estland	X		Sudan				
X	ES	Spanien	X	SE	Schweden				
X	FI	Finnland	X	SG	Singapur				
X	GB	VereinigtesKönigreich	X	SI	Slowenien				
X	GD	Grenada	図	SK	Slowakei				
X	GE	Georgien	X		SierraLeone				
×		Ghana	X	TJ	Tadschikistan				
X		Gambia	X		Turkmenistan				
X	HR	Kroatien	X		Türkei				
X	HU	Ungarn							
X		Indonesien	X		TrinidadundTobago				
X		Israel	X		Ukraine				
X		Indien	X		Uganda				
X		Island	X	02	VereinigteStaaten von Amerika				
			(CP)	*	TT 1 4 4 4				
N X		Japan	X		Usbekistan				
X		Kenia	X		Vietnam				
(S)		Kirgisistan	X	YU	Jugoslawien				
X	r.r	Demokratische Volksrepublik Korea	X	ZA	Südafrika				
<u></u>	L/D	D LUI-77	X		Simbabwe				
X		RepublikKorea	Käs	chenfu	rdieBestimmungvonStaaten,diedemPCTnachder				
X		Kasachstan	ver	offentlic	chungdieses Formblatts beigetreten sind:				
×		SaintLucia		14	Vereinigte Republik Tansania				
X		SriLanka		MA	Marokko				
Erkli	rung	bzgl. vorsorglicher Bestimmungen: Zusätzlich zu	deno	hen gen	annten Restimmungen nimmt des Anmaldes				
Besti: dem\	mmun Jorbeh	osalz bauch alle anderen nachdem PCT zulässigen Best gen, die von dieser Erklärung ausgenommen sind. Der A alteiner Bestätigungstehen und jedez usätzliche Bestim igt wurde, nach Ablauf dieser Frist als vom Anmelder hdie Einreichungeiner Mitteilung, inder diese Bestimm	ımmı Inme mune	ingen v ldererki dievor	ormit Ausnahmeder im Zusatzfeld genannten lätt, daßdiesezusätzlichen Bestimmungen unter - Ablauf von 15 Monatenablem Prioritäte det um				
done	antätia	amanachiika Die Postäti Ol A	ariga	Regeo	en wir a, una aie Zaniungaer Bestimmungs-und				

BlattNr 3

		BlattNr			
FeldNr.VI PRIORITÄTS.	ANSPRUCH		Weiter	ePrioritätsansprüchesindi	imZusatzfeldangegeben
Anmeldedatum derfrüherenAnmeldung	Aktenzeichen derfrüheren Anmeldung			IstdiefrühereAnmeldun	geine:
(Tag/Monat/Jahr)	derrunerenAnmeidung	nationaleAn Sta			internationaleAnmeldun
Zeile(1) (18.01.1999) 18 JAN 1999	19901761.1	DE		regionalesAm	Anmeldeamt
Zeile(2) 29 APR 1999 (29.04.1999)	19926457.0	DE			
Zeile(3)					
Das Anmeldeamt wird ersucht bezeichneten früheren Anmel- dem Amteingereicht worden is * Falls es sich bei der früheren Anm Mitgliedstaat der Pariser Verbandsül	aufigen) zu erstellen und der et(sind), das für die Zwecke di ieldung um eine ARIPO-Anm bereinkunst zum Schutz des	n internationalen eserinternationa eldung handelt, sa gewerblichen Eige	Büro zu übe		rühere Anmeldung(en) bei Staat angegeben werden, der neldung eingereicht wurde.
relant.vii INTERNATIO	NALERECHERCHEN	BEHÖRDE			
Wahl der internationalen Recherchen (falls zwei oder mehr als zwei internatio behörden für die Ausführung der internat zuständigsind, geben Siedievon Ihnenge w der Zweibuchstaben-Codekannbenutzw	onale Recherchen- tionalen Recherche bear ählteBehördean:	rag auf Nutzung d iere Recherche (fa ntragtodervonihrdi um (Tag/Monat/	ırchgeführt		nalenRecherchenbehörde
ISA / EP		-	,		Staat (oder regionales Amt)
FeldNr.VII I KONTROLLIS	TEEINREICHUNGSS	PRACHE			
DieseinternationaleAnmeldungen diefolgendeAnzahlvonBlättern	thält Dieserinternation	nalenAnmeldun	g liegend	ienachstehendangekreuz	tenUnterlagen bei:
Antrag : 3	1. 🗶 Blattfürdi	eGebührenbere	chnung		
Beschreibung(ohne	2. Gesonder				
Sequenzprotokollteil) : 61	3. Kopiedera	allgemeinenVol	lmacht;Ak	tenzeichen(fallsvorhande	en):
Ansprüche : 8	4. Begründu				
Zusammenfassung : 1	5. Prioritätst folgendeZ	peleg(e),inFeldl eilennummerge	Nr.VIdurci kennzeich	h net:	
Zeichnungen : 6				met. eldungindiefolgendeSpra	naha.
Sequenzprotokollteil derBeschreibung :	7. Gesonderte	Angaben zu hint	erlegten M	ikroorganismen oder andere	um biologischen Metaria
	— 8. 🔲 Protokolld	erNucleotid-un	d/oderAm	inosäuresequenzenincom	muterlesharerForm
Blattzahlinsgesamt : 79	9. 🗷 Sonstige(e	inzelnaussühren): Schect	Nr. 3000022382885	-paronesouren onn
Abbildung der Zeichnungen, die mit der Zusammenfassung veröffentlicht werden soll (Nr.): Fig	Spra inten	che, inderdie nationale Anmeld reicht wird:			
FeldNr.IX UNTERSCHRIFT	DESANMELDERSON	FRDESANW	27 14	····	
Der Name jeder unterzeichnenden P aus dem Antrag ergibt, in welcher München, 04. Japuar 2000	arrow ist noben den II.	. 1 . 6	holen, und	es ist anzugeben, sofern	sich dies nicht eindeutig
J. (2)					
Gerhard Hartwich					
. Datum des tatsächlichen Eingang internationalen Anmeldung:		meldeamtauszu (0		00) 0 7 JAN 2000	2. Zeichnungen
. Geändertes Eingangsdatumaufgr fristgerecht eingegangener Unter zur Vervollständigung dieserintet	lagen oder Zoichning			y = 31111 2000	einge- gangen:
DatumdesfristgerechtenEingangs RichtigstellungennachArtikell1	derangeforderten (2)PCT:				nichtein- gegangen:
. InternationaleRecherchenbehörd (fallszweiodermehrzuständigsind	d): ISA/	6.	Überm Zahlun	ittlungdesRecherchenexe gderRecherchengebühra	emplarsbiszur uufgeschoben
Datum des Eingangs des Aktenexer beimInternationalen Büro:		onalenBüroaus	zufüllen		
emblastPCT/P.O/101/Jeters Planty					

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum Internationales Büro





(43) Internationales Veröffentlichungsdatum 20. Juli 2000 (20.07.2000)

PCT

(10) Internationale Veröffentlichungsnummer WO 00/42217 A3

(51) Internationale Patentklassifikation?: C07H 21/00

C12Q 1/68,

(21) Internationales Aktenzeichen:

PCT/EP00/00084

(22) Internationales Anmeldedatum:

7. Januar 2000 (07.01.2000)

(25) Einreichungssprache:

Deutsch

(26) Veröffentlichungssprache:

Deutsch

(30) Angaben zur Priorität:

199 01 761.1

18. Januar 1999 (18.01.1999) DE

199 26 457.0

29. April 1999 (29.04.1999) Di

- (72) Erfinder; und
- (75) Erfinder/Anmelder (nur für US): HARTWICH, Gerhard [DE/DE]; Nibelungenstrasse 10, D-80639 München (DE)

(81) Bestimmungsstaaten (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Bestimmungsstaaten (regional): ARIPO-Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI-Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

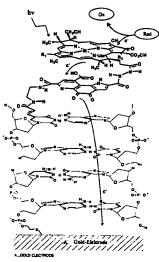
Veröffentlicht:

Mit internationalem Recherchenbericht.

[Fortsetzung auf der nächsten Seite]

(54) Title: METHOD FOR ELECTROCHEMICALLY DETECTING NUCLEIC ACID-OLIGOMER HYBRIDISATION EVENTS

(54) Bezeichnung: VERFAHREN ZUR ELEKTROCHEMISCHEN DETEKTION VON NUKLEINSÄURE -OLIGOMER- HYBRIDISIERUNGSEREIGNISSEN



(57) Abstract: The invention relates to a method for electrochemically detecting sequence-specific nucleic acid-oligomer hybridisation events. DNA/RNA/PNA oligomer single strands which are bound to a conductive surface at one end and linked to a redoxactive unit at the other, free end, serve as a hybridisation matrix (probe). A proportion of the single strand oligonucleotides are hybridised by treatment with the oligonucleotide solution (target) being tested, with the result that the electrical communication between the conductive surface and the redoxactive unit, which is initially non- or barely existent, is increased. This enables a hybridisation event to be detected using electrochemical methods such as voltammetry, amperometry or conductance measurement.

[Fortsetzung auf der nächsten Seite]



WO 00/42217 A3



(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 30. November 2000

Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

⁽⁵⁷⁾ Zusammenfassung: Die vorliegende Erfindung betrifft ein Verfahren zur elektrochemischen Detektion von sequenzspezifischen Nukleinsäure-Oligomer-Hybridisierungsereignissen. Dabei dienen DNA-/RNA-/PNA-Oligomer-Einzelstränge, die mit einem Ende an einer leitfähigen Oberfläche gebunden und am anderen, freien Ende mit einer redoxaktiven Einheit verknüpft sind, als Hybridisierungsmatrix (Sonde). Durch Behandlung mit der zu untersuchenden Oligonukleotid-Lösung (Target) wird ein Teil der Einzelstrang-Oligonukleotide hybridisiert, wodurch die ursprünglich nicht oder nur schwach vorhandene elektrische Kommunikation zwischen der leitfähigen Oberfläche und der redoxaktiven Einheit erhöht wird. Somit wird die Detektion eines Hybridisierungsereignisses durch elektrochemische Verfahren wie Voltametrie, Amperometrie oder Leitfähigkeitsmessung ermöglicht.

INTERNATIONAL SEARCH REPORT

nal Application No PCI/EP 00/00084

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C1201/68 C07H21/00

C. DOCUMENTS CONSIDERED TO BE RELEVANT

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
χ	US 5 770 369 A (FRASER SCOTT E ET AL) 23 June 1998 (1998-06-23) the whole document	1-55
X	WO 98 20162 A (GOZIN MICHAEL ;YU CHANGJUN (US); KAYYEM JON F (US); CLINICAL MICRO) 14 May 1998 (1998-05-14) the whole document	1-5, 21-23, 29,30
X	US 5 622 946 A (HARRIMAN ANTHONY M ET AL) 22 April 1997 (1997-04-22) example 4	1-5, 21-23
X	DE 42 16 696 A (DEUTSCHE AEROSPACE) 28 October 1993 (1993-10-28) the whole document	1-5

Y Further documents are listed in the continuation of box C.	Patent family members are listed in annex.
Special categories of cited documents :	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention
"L" document which may throw doubts on priority claim(s) or	cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention
"O" document referring to an oral disclosure, use, exhibition or other means	cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled
P document published prior to the international filing date but	in the art.
later than the priority date claimed	"&" document member of the same patent family
Date of the actual completion of the international search	Date of mailing of the international search report
6 September 2000	13/09/2000
Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Reuter, U

INTERN CONAL SEARCH REPORT

Honal Application No
PCI/EP 00/00084

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT Category * Citation of document, with indication,where appropriate, of the relevant passages A US 5 312 527 A (MIKKELSEN SUSAN R FT AL)	Relevant to claim No.
A US 5 212 527 A (MIVVELSEN CHEAN D. FT. AL.)	
A US 5 312 527 A (MIKKELSEN SUSAN R ET AL) 17 May 1994 (1994-05-17) the whole document	1-55
A KATZ E ET AL: "ELECTROCHEMICAL STUDY OF PYRROLOQUINOLINE QUINONE COVALENTLY IMMOBILIZED AS A MONOLAYER ONTO A CYSTAMINE-MODIFIED GOLD ELECTRODE" JOURNAL OF ELECTROANALYTICAL CHEMISTRY AND INTERFACIAL ELECTROCHEMISTRY, NL, ELSEVIER, AMSTERDAM, vol. 367, 1994, pages 59-70, XP000672483 ISSN: 0022-0728 the whole document	1-55
A WO 98 31839 A (HARVARD COLLEGE) 23 July 1998 (1998-07-23) the whole document	1-55
A EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25 March 1998 (1998-03-25) the whole document	1-55
P,X WO 99 51778 A (BARTON JACQUELINE ;HILL MICHAEL (US); KELLEY SHANA (US); CALIFORNI) 14 October 1999 (1999-10-14) the whole document	1-5, 21-23, 29,30
E WO 00 31101 A (HARTWICH GERHARD ;HELLER ADAM (US)) 2 June 2000 (2000-06-02) the whole document	1-55

INTERNA "NAL SEARCH REPORT

Information on patent family members

nal Application No

Patent document cited in search report	Publication date		ent family ember(s)	Publication date
US 5770369	A 23-06-1998	US US	5824473 A 5591578 A	20-10-1998 07-01-1997
		US	6071699 A	06-06-2000
		US	5952172 A	14-09-1999
		AU	6166296 A	30-12-1996
		EP WO	0871642 A 9640712 A	21-10-1998
		AU	9640712 A 703329 B	19-12-1996 25-03-1999
		AU	1215295 A	27-06-1995
		CA	2178618 A	15-06-1995
		EP	0733058 A	25-09-1996
		JP	9506510 T	30-06-1997
		WO	9515971 A	15-06-1995
		US US	5780234 A 5705348 A	14-07-1998
		US	6087100 A	06-01-1998 11-07-2000
W0 9820162	A 14-05-1998		6096273 A	
40 JOEOTOE /	17 03 1330		6090273 A	01-08-2000 18-07-2000
			5196798 A	29-05-1998
			0939762 A	08-09-1999
US 5622946	A 22-04-1997		5457183 A	10-10-1995
			6072038 A	06-06-2000
			5583220 A 5580543 A	10-12-1996
			5587371 A	03-12-1996 2 4- 12-1996
			5632970 A	27-05-1997
		US	5801229 A	01-09-1998
		US	5969111 A	19-10-1999
			5888997 A	30-03-1999
			6069140 A	30-05-2000
		AU AU	683316 B 8075694 A	06-11-1997 04-05-1995
			2173319 A	20-04-1995
			0724457 A	07-08-1996
		JP	9508616 T	02-09-1997
		NO	961436 A	11-06-1996
		SG	48392 A	17-04-1998
			9510307 A 5599923 A	20-04-1995 04-02-1997
DE 4216696 A	28 - 10-1993	*		
			9321530 A 	28-10-1993
US 5312527 A	17-05-1994	NONE		
WO 9831839 A	23-07-1998		5926598 A	07-08-1998
		EP	0981643 A 	01-03-2000
EP 0831327 A	25-03-1998	DE 19	9639169 A	02-04-1998
			2216105 A 0130247 A	24-03-1998 19-05-1998
			6057120 A	02-05-2000
WO 9951778 A	14-10-1999	AU	3550699 A	25-10-1999
WO 0031101 A	02-06-2000	DE 19	9921940 A	15-06-2000

INTERNATIONALER PECHERCHENBERICHT

Onales Aktenzeichen
PLI/EP 00/00084

a. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES IPK 7 C12Q1/68 C07H21/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Massifikationssystem und Klassifikationssymbole) IPK 7 C120 C07H

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegnffe)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

Kategorie®	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.				
X	US 5 770 369 A (FRASER SCOTT E ET AL) 23. Juni 1998 (1998-06-23) das ganze Dokument	1-55				
X	WO 98 20162 A (GOZIN MICHAEL ;YU CHANGJUN (US); KAYYEM JON F (US); CLINICAL MICRO) 14. Mai 1998 (1998-05-14) das ganze Dokument	1-5, 21-23, 29,30				
X	US 5 622 946 A (HARRIMAN ANTHONY M ET AL) 22. April 1997 (1997-04-22) Beispiel 4	1-5, 21-23				
X	DE 42 16 696 A (DEUTSCHE AEROSPACE) 28. Oktober 1993 (1993-10-28) das ganze Dokument/	1-5				

Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen	X Siehe Anhang Patentfamilie					
 Besondere Kategorien von angegebenen Veröffentlichungen: 'A' Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist 'E' älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist 'L' Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt) 'O' Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht 'P' Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist 	 "T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständris des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist "X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden "Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist "&" Veröffentlichung, die Mitglied derselben Patentfamilie ist 					
Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts					
6. September 2000	13/09/2000					
Name und Postanschrift der Internationalen Recherchenbehörde Europäisches Patentamt, P.B. 5818 Patentlaan 2 Nt. – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bevolmächtigter Bediensteter Reuter, U					

INTERNATIONALEF CECHERCHENBERICHT

PCT/EP 00/00084

C.(Fortsetzu	ng) ALS WESENTLICH ANGESEHENE UNTERLAGEN	00/00084
Kategone'	Bezeichnung der Veröffentlichung, soweit erfordenlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	US 5 312 527 A (MIKKELSEN SUSAN R ET AL) 17. Mai 1994 (1994-05-17) das ganze Dokument	1-55
A	KATZ E ET AL: "ELECTROCHEMICAL STUDY OF PYRROLOQUINOLINE QUINONE COVALENTLY IMMOBILIZED AS A MONOLAYER ONTO A CYSTAMINE-MODIFIED GOLD ELECTRODE" JOURNAL OF ELECTROANALYTICAL CHEMISTRY AND INTERFACIAL ELECTROCHEMISTRY, NL, ELSEVIER, AMSTERDAM, Bd. 367, 1994, Seiten 59-70, XP000672483 ISSN: 0022-0728 das ganze Dokument	1-55
١	WO 98 31839 A (HARVARD COLLEGE) 23. Juli 1998 (1998-07-23) das ganze Dokument	1-55
١	EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25. März 1998 (1998-03-25) das ganze Dokument	1–55
, x	WO 99 51778 A (BARTON JACQUELINE ;HILL MICHAEL (US); KELLEY SHANA (US); CALIFORNI) 14. Oktober 1999 (1999-10-14) das ganze Dokument	1-5, 21-23, 29,30
	WO 00 31101 A (HARTWICH GERHARD; HELLER ADAM (US)) 2. Juni 2000 (2000-06-02) das ganze Dokument	1-55

INTERNATIONALER STCHERCHENBERICHT

Angaben zu Veröffentlichungen, di.

selben Patentfamilie gehören

I. iales Aktenzeichen PCI/EP 00/00084

	Recherchenberk hrtes Patentdoki		Datum der Veröffentlichung	^	Aitglied(er) der Patentfamilie	Datum der Veröffentlichung
US	5770369	Α	23-06-1998	US	5824473 A	20-10-1998
			,	ÜS	5591578 A	07-01-1997
				US	6071699 A	06-06-2000
				US	5952172 A	14-09-1999
				AU	6166296 A	30-12-1996
				ΕP	0871642 A	21-10-1998
				WO	9640712 A	19-12-1996
				AU	703329 B	25-03-1999
				AU	1215295 A	27-06-1995
				CA	2178618 A	15-06-1995
				EP	0733058 A	25-09-1996
				ĴΡ	9506510 T	30-06-1997
				WO.	9515971 A	15-06-1995
				ÜS	5780234 A	14-07-1998
				US	5705348 A	06-01-1998
				US	6087100 A	
					A	11-07-2000
WO	9820162	Α	14-05-1998	US	6096273 A	01-08-2000
				US	6090933 A	18-07-2000
				AU	5196798 A	29-05-1998
				EP	0939762 A	08-09-1999
US	5622946	A	22-04-1997	US	5457183 A	10-10-1995
				US	6072038 A	06-06-2000
				ÜS	5583220 A	10-12-1996
				ÜS	5580543 A	03-12-1996
				ÜŠ	5587371 A	24-12-1996
				ÜS	5632970 A	27-05-1997
				US	5801229 A	01-09-1998
				US	5969111 A	19-10-1999
				US	5888997 A	30-03-1999
				US	6069140 A	30-05-2000
				AU	683316 B	06-11-1997
				AU	8075694 A	04-05-1995
				CA	2173319 A	20-04-1995
				EP	0724457 A	07-08-1996
				ĴΡ	9508616 T	
				NO	961436 A	02-09-1997
				SG	48392 A	11-06-1996 17-04-1998
				WO	9510307 A	1/-04-1998
				MO	2010005 V	
				US		04-02-1997
DE	4216696 	A	28-10-1993 	WO	9321530 A	28-10-1993
US	5312527	A	17-05-1994	KEIN	IE	
WO	9831839	Α	23-07-1998	AU	5926598	07-08-1998
				EP	0981643 A	01-03-2000
EP	0831327	Α	25-03-1998			02-04-1998
				CA	2216105 A	
				JP	10130247 A	19-05-1998
					6057120 A	02-05-2000
WO	9951778			ΑU		25-10-1999
	0021101	Λ	02-06-2000			15-06-2000

PCT

WELTORGANISATION FÜR GEISTIGES EIGENTUM Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 7:
C12Q 1/68

A2
(11) Internationale Veröffentlichungsnummer: WO 00/42217
(43) Internationales
Veröffentlichungsdatum: 20, Juli 2000 (20,07,00)

(21) Internationales Aktenzeichen: PCT/EP00/00084

(22) Internationales Anmeldedatum: 7. Januar 2000 (07.01.00)

(30) Prioritätsdaten:

199 01 761.1 18. Januar 1999 (18.01.99) DE 199 26 457.0 29. April 1999 (29.04.99) DE

(71)(72) Anmelder und Erfinder: HARTWICH, Gerhard [DE/DE]; Nibelungenstrasse 10, D-80639 München (DE). (81) Bestimmungsstaaten: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht

Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(54) Title: METHOD FOR ELECTROCHEMICALLY DETECTING NUCLEIC ACID-OLIGOMER HYBRIDISATION EVENTS

(54) Bezeichnung: VERFAHREN ZUR ELEKTROCHEMISCHEN DETEKTION VON NUKLEINSÄURE -OLIGOMER- HYBRI-DISIERUNGSEREIGNISSEN

(57) Abstract

The invention relates to a method for electrochemically detecting sequence-specific nucleic acid-oligomer hybridisation events. DNA/RNA/PNA oligomer single strands which are bound to a conductive surface at one end and linked to a redoxactive unit at the other, free end, serve as a hybridisation matrix (probe). A proportion of the single strand oligonucleotides are hybridised by treatment with the oligonucleotide solution (target) being tested, with the result that the electrical communication between the conductive surface and the redoxactive unit, which is initially non- or barely existent, is increased. This enables a hybridisation event to be detected using electrochemical methods such as voltammetry, amperometry or conductance measurement.

(57) Zusammenfassung

Erfindung Die vorliegende betrifft Verfahren ein elektrochemischen Detektion von sequenzspezifischen Nukleinsäure-Oligomer-Hybridisierungsereignissen. Dabei dienen DNA-/RNA-/PNA-Oligomer-Einzelstränge, die mit einem Ende an einer leitfähigen Oberfläche gebunden und am anderen, freien Ende mit einer redoxaktiven Einheit verknüpft sind, als Hybridisierungsmatrix (Sonde). Durch Behandlung mit der zu untersuchenden Oligonukleotid-Lösung (Target) wird ein Teil der Einzelstrang-Oligonukleotide hybridisiert, wodurch die ursprünglich nicht oder nur schwach vorhandene elektrische Kommunikation zwischen der leitfähigen Oberfläche und der redoxaktiven Einheit erhöht wird. Somit wird die Detektion eines Hybridisierungsereignisses durch elektrochemische Verfahren wie Voltametrie, Amperometrie oder Leitfähigkeitsmessung ermöglicht.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AL	Albanien	ES	Spanien	LS	Lesotho	SI	Slowenien
AM	Armenien	FI	Finnland	LT	Litauen	SK	Slowakei
AT	Österreich	FR	Frankreich	LU	Luxemburg	SN	Senegal
AU	Australien	GA	Gabun	LV	Lettland	SZ	Swasiland
AZ	Aserbaidschan	GB	Vereinigtes Königreich	MC	Monaco	TD	Tschad
BA	Bosnien-Herzegowina	GE	Georgien	MD	Republik Moldau	TG	
BB	Barbados	GH	Ghana	MG	Madagaskar	TJ	Togo Tadschikistan
BE	Belgien	GN	Guinea	MK	Die ehemalige jugoslawische	TM	
BF	Burkina Faso	GR	Griechenland		Republik Mazedonien	TR	Turkmenistan
BG	Bulgarien	HU	Ungam	ML	Mali	TT	Türkei
BJ	Benin	ΙE	Irland	MN	Mongolei	UA	Trinidad und Tobago
BR	Brasilien	IL	Israel	MR	Mauretanien		Ukraine
BY	Belarus	IS	Island	MW	Malawi	UG	Uganda
CA	Kanada	IT	Italien	MX	Mexiko	US	Vereinigte Staaten von
CF	Zentralafrikanische Republik	JР	Japan	NE	Niger		Amerika
CG	Kongo	KE	Kenia	NL	Niederlande	UZ VN	Usbekistan
CH	Schweiz	KG	Kirgisistan	NO	Norwegen	YU	Vietnam
CI	Côte d'Ivoire	KP	Demokratische Volksrepublik	NZ	Neuseeland		Jugoslawien
CM	Kamerun		Korea	PL	Polen	zw	Zimbabwe
CN	China	KR	Republik Korea	PT	Portugal		
CU	Kuba	ΚZ	Kasachstan	RO	Rumānien		
CZ	Tschechische Republik	LC	St. Lucia	RU	Russische Föderation		
DE	Deutschland	LI	Liechtenstein	SD	Sudan		
DK	Dänemark	LK	Sri Lanka	SE	Schweden		
EE	Estland	LR	Liberia	SG	Singapur		
					O.k.		
1							

WO 00/42217 PCT/EP00/00084

Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybridisierungsereignissen

Technisches Gebiet

Die vorliegende Erfindung betrifft ein modifiziertes Nukleinsäure-Oligomer, sowie ein Verfahren zur elektrochemischen Detektion von sequenzspezifischen Nukleinsäure-Oligomer-Hybridisierungsereignissen.

Stand der Technik

Zur Sequenzanalyse von DNA und RNA, z. B. in der Krankheitsdiagnose, bei toxikologischen Testverfahren, in der genetischen Forschung und Entwicklung, sowie auf dem Agrar- und pharmazeutischen Sektor, werden im allgemeinen gelektrophoretische Verfahren mit autoradiographischer og 30 optischer Detektion verwendet.

Beim wichtigsten gel-elektrophoretischen Verfahren mit optischer Detektion, dem Sanger-Verfahren wird eine DNA enthaltende Lösung in vier Ansätze aufgeteilt. Zur Unterscheidung der vier Ansätze ist der Primer (komplementäre Startsequenz zur Replikation) jedes Ansatzes mit je einem bei verschiedener Wellenlänge emitierenden Fluoreszenzfarbstoff kovalent modifiziert. Ausgehend vom Primer wird jeder Ansatz durch DNA-Polymerase I enzymatisch repliziert. Neben den dazu nötigen Desoxyribonucleosid-Triphosphaten der Basen A (Adenin), T (Thymin), C (Cytosin), und G (Guanin) enthält jedes Reaktionsgemisch noch genügend 2',3'-Didesoxyanalogon eines dieser Nukleosidtriphosphate als Stopbase (je eine der 4 möglichen Stopbasen pro Ansatz), um die Replikation an allen möglichen Bindungsstellen zu stoppen. Nach Vereinigung der vier Ansätze entstehen replizierte DNA-Fragmente aller Längen mit stopbasenspezifischer Fluoreszenz, die gelelektrophoretisch der Länge nach sortiert und durch Fluoreszenz-Spektroskopie charakterisiert werden können.

Ein anderes optisches Detektionsverfahren basiert auf der Anlagerung von Fluoreszenzfarbstoffen wie z. B. Ethidiumbromid an Oligonukleotide. Im Vergleich zur freien Lösung des Farbstoffs ändert sich die Fluoreszenz solcher Farbstoffe bei Assoziation mit doppelsträngiger DNA oder RNA drastisch und kann deshalb zum Nachweis hybridisierter DNA oder RNA verwendet werden.

WO 00/42217 PCT/EP00/00084

Bei der radioaktiven Markierung wird ³²P in das Phosphatgerüst der Oligonukleotide eingebaut, wobei ³²P gewöhnlich am 5'-Hydroxylende durch Polynukleotid-Kinase addiert wird. Die markierte DNA wird anschließend an jeweils einem der vier Nukleotidtypen bevorzugt gespalten und zwar unter definierten Bedingungen, so daß pro Kette durchschnittlich eine Spaltung erfolgt. Damit liegen im Reaktionsgemisch für einen bestimmten Basentyp Ketten vor, die sich von der ³²P-Markierung bis zur Position dieser Base erstrecken (bei mehrfachem Auftreten der Base erhält man entsprechend Ketten unterschiedlicher Länge). Die vier Fragmentgemische werden anschließend auf vier Bahnen gel-elektrophoretisch aufgetrennt. Danach wird vom Gel ein Autoradiogramm angefertigt, an dem die Sequenz unmittelbar abgelesen werden kann.

Vor einigen Jahren wurde ein weiteres, auf optischer (oder autoradiographischer) Detektion beruhendes Verfahren zur DNA-Sequenzierung entwickelt, nämlich die Sequenzierung durch Oligomer-Hybridisierung (vgl. z. B. Drmanac et al., Genomics 4, (1989), S. 114-128 oder Bains et al., Theor. Biol. 135, (1988), S. 303-307). Bei diesem Verfahren wird ein vollständiger Satz kurzer Oligonukleotide Nukleinsäure-Oligomere (Sonden-Oligonukleotide), z. B. alle 65536 möglichen Kombinationen der Basen A, T, C und G eines Oligonukleotid-Oktamers auf ein Trägermaterial gebunden. Die Anbindung geschieht in einem geordneten Raster aus 65536 Test-Sites, wobei jeweils eine größere Menge einer Oligonukleotid-Kombination ein Test-Site definieren und die Position jeder einzelnen Test-Site (Oligonukleotid-Kombination) bekannt ist. Auf solch einer Hybridisierungsmatrix, dem Oligomer-Chip, wird ein DNA-Fragment, dessen Sequenz man ermitteln will (das Target), mit Fluoreszenzfarbstoff (oder ³²P) markiert und unter Bedingungen, die nur eine spezifische Doppelstrangbildung erlauben, hybridisiert. Dadurch bindet das Target DNA-Fragment nur an die Nukleinsäure-Oligomere (im Beispiel an die Oktamere), deren komplementäre Sequenz exakt einem Teil (einem Oktamer) seiner eigenen Sequenz entspricht. Durch optische (oder autoradiographische) Detektion der Bindungsposition des hybridisierten DNA-Fragments werden damit alle im Fragment vorhandenen Nukleinsäure-Oligomersequenzen (Oktamersequenzen) bestimmt. Aufgrund der Überlappung benachbarter Nukleinsäure-Oligomersequenzen kann durch geeignete mathematische Algorithmen die fortlaufende Sequenz des DNA-Fragments bestimmt werden. Die Vorteile dieses Verfahrens liegen unter anderem in der Miniaturisierung der Sequenzierung und damit in der enormen Datenmenge, die gleichzeitig in einem Arbeitsgang erfaßt wird. Daneben kann auf Primer und auf das gel-elektrophoretische Auftrennen der DNA-Fragmente verzichtet werden. Beispielhaft ist dieses Prinzip in Figur 1 für ein 13 Basen langes DNA-Fragment gezeigt.

Die Verwendung radioaktiver Markierungen bei der DNA-/RNA-Sequenzierung ist mit mehreren Nachteilen verbunden, wie z. B. aufwendige, gesetzlich vorgeschriebene Sicherheitsvorkehrungen beim Umgang mit radioaktiven Materialien, die Strahlenbelastung, das begrenzte räumliche Auflösungsvermögen (maximal 1mm²) und eine Sensitivität, die nur dann hoch ist, wenn die Strahlung der radioaktiven Fragmente entsprechend lange (Stunden bis Tage) auf einen Röntgenfilm einwirkt. Es kann zwar die räumliche Auflösung durch zusätzliche Hard- und Software erhöht und die Detektionszeit durch die Verwendung von β -Scannern verkürzt werden, beides ist jedoch mit erheblichen zusätzlichen Kosten verbunden.

Die Fluoreszenzfarbstoffe, die üblicherweise zur Markierung der DNA verwendet werden, sind zum Teil (z. B. Ethidiumbromid) mutagen und erfordern, ebenso wie die Anwendung der Autoradiographie, entsprechende Sicherheitsvorkehrungen. In fast allen Fällen erfordert die Verwendung optischer Detektion den Gebrauch von einem oder mehreren Lasersystemen und somit geschultes Personal und entsprechende Sicherheitsvorkehrungen. Die eigentliche Detektion der Fluoreszenz erfordert zusätzliche Hardware, wie z. B. optische Bauelemente zur Verstärkung und, bei verschiedenen Anregungs- und Abfragewellenlängen wie im Sanger-Verfahren, ein Kontrollsystem. Abhängig von den benötigten Anregungswellenlängen und der gewünschten Detektionsleistung können somit erhebliche Investitionskosten entstehen. Bei der Sequenzierung durch Hybridisierung auf dem Oligomer-Chip ist die Detektion noch (kosten)aufwendiger, da, neben dem Anregungssystem, zur 2-dimensionalen Detektion der Fluoreszenzspots hochauflösende CCD-Kameras (Charge Coupled Device Kameras) benötigt werden.

Obwohl es also quantitative und extrem sensitive Methoden zur DNA-/RNA-Sequenzierung gibt, sind diese Methoden zeitaufwendig, bedingen aufwendige Probenpräparation und teure Ausstattung und sind im allgemeinen nicht als transportable Systeme verfügbar.

Darstellung der Erfindung

Aufgabe der vorliegenden Erfindung ist es deshalb, eine Vorrichtung und ein Verfahren zur Detektion von Nukleinsäure-Oligomer-Hybriden zu schaffen, welche die Nachteile des Standes der Technik nicht aufweisen.

Diese Aufgabe wird erfindungsgemäß durch das modifizierte Nukleinsäure-Oligomer gemäß unabhängigem Patentanspruch 1, durch das Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers gemäß unabhängigem Anspruch 21, durch die modifizierte leitfähige Oberfläche gemäß unabhängigem Patentanspruch 29, das Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche gemäß unabhängigem Patentanspruch 43 und ein Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybridisierungsereignissen gemäß unabhängigen Patentanspruch 48 gelöst.

Im Rahmen der vorliegenden Erfindung werden die folgenden Abkürzungen und Begriffe benutzt:

G	e	n	e	ti	k

DNA	Desoxyribonukleinsäure			
RNA	Ribonukleinsäure			
PNA	Peptidnukleinsäure (synthetische DNA oder RNA, bei der die Zucker-Phosphat Einheit durch eine Aminosäure ersetzt ist. Bei Ersatz der Zucker-Phosphat Einheit durch die -NH-(CH ₂) ₂ -N(COCH ₂ -Base)-CH ₂ CO- Einheit hybridisiert PNA mit DNA.)			
Α	Adenin			
G	Guanin			
С	Cytosin			
T	Thymin			
U	Uracil			
Base	A, G, T, C oder U			
Вр	Basenpaar			
Nukleinsäure	wenigstens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- (z. B. Cytosin, Thymin oder Uracil) oder Purin-Basen (z. B. Adenin oder Guanin). Der Begriff Nukleinsäure bezieht sich auf ein beliebiges "Rückgrat" der kovalent verbundenen Pyrimidinoder Purin-Basen, wie z. B. auf das Zucker-Phosphat Rückgrat der DNA, cDNA oder RNA, auf ein Peptid-Rückgrat der PNA oder auf analoge Strukturen (z. B. Phosphoramid-, Thio-Phosphat- oder Dithio-Phosphat-Rückgrat).			

Wesentliches Merkmal einer Nukleinsäure im Sinne der vorliegenden Erfindung ist, daß sie natürlich vorkommende cDNA oder RNA sequenzspezifisch binden kann.

Nukleinsäure-Oligomer Nukleinsäure nicht näher spezifizierter Basenlänge (z. B. Nukleinsäure-Oktamer: eine Nukleinsäure mit beliebigem Rückgrat, bei dem 8 Pyrimidin- oder Purin-Basen kovalent aneinander gebunden sind).

Oligomer

Äquivalent zu Nukleinsäure-Oligomer.

Oligonukleotid

Äquivalent zu Oligomer oder Nukleinsäure-Oligomer, also z. B. ein DNA, PNA oder RNA Fragment nicht näher spezifizierter Basenlänge.

Oligo

Abkürzung für Oligonukleotid.

Primer

Start-Komplementär-Fragment eines Oligonukleotids, wobei die Basenlänge des Primers nur ca. 4-8 Basen beträgt. Dient als Ansatzpunkt für die enzymatische Replikation des Oligonukleotids.

Mismatch

Zur Ausbildung der Watson Crick Struktur doppelsträngiger Oligonukleotide hybridisieren die beiden Einzelstränge derart, daß die Base A (bzw. C) des einen Strangs mit der Base T (bzw. G) des anderen Strangs Wasserstoffbrücken ausbildet (bei RNA ist T durch Uracil ersetzt). Jede andere Basenpaarung bildet keine Wasserstoffbrücken aus, verzerrt die Struktur und wird als "Mismatch" bezeichnet.

SS

single strand (Einzelstrang)

ds

double strand (Doppelstrang)

Photoinduzierbar und chemisch induzierbar redoxaktive Einheiten

redoxaktive Einheit

photoinduzierbar redoxaktive Einheit oder chemisch induzierbar redoxaktive Einheit

Elektron-Donor

Der Begriff Elektron-Donor bezeichnet im Rahmen der vorliegenden Erfindung einen Bestandteil einer photoinduzierbar redoxaktiven Einheit bzw. einer chemisch induzierbar redoxaktiven Einheit. Bei einem Elektron-Donor handelt es sich um ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände ein Elektron an einen Elektron-

Akzeptor transferieren kann. Ein solcher äußerer Umstand ist z. B. die Lichtabsorption durch den Elektron-Donor oder -Akzeptor einer photoinduzierbar redoxaktiven Einheit. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der Elektron-Donor "D" an den/einen Elektron-Akzeptor "A" ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D*A- aus oxidiertem Donor und reduziertem Akzeptor. Ein weiterer solcher äußerer Umstand kann z. B. die Oxidation oder Reduktion des Elektron-Donors oder -Akzeptors der chemisch induzierbar redoxaktiven Einheit durch ein externes Oxidations- oder Reduktionsmittel sein, also z. B. die Übertragung eines Elektrons auf den Elektron-Donor durch ein Reduktionsmittel bzw. die Abgabe eines Elektrons durch den Elektron-Akzeptor an ein Oxidationsmittel sein. Diese Oxidations- bzw. Reduktionsmittel können sowohl externe redoxaktive Substanzen sein, d. h. sie sind nicht kovalent mit der redoxaktiven Einheit, dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbunden, stehen aber mit diesen, z. B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung, in Kontakt oder sie sind kovalent mit dem Nukleinsäure-Oligomer verbunden, wobei das Oxidations-Reduktionsmittel an einer Stelle des Nukleinsäure-Oligomers kovalent angebunden ist, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- oder Purin-Basen von der kovalenten Anbindungstelle der redoxaktiven Einheit entfernt ist, bevorzugt dem der Modifikation mit redoxaktiver Einheit entgegengesetzten Ende des Oligonukleotids in der Nähe der leitfähigen Oberfläche. Die Fähigkeit als Elektron-Donor oder -Akzeptor zu wirken ist relativ, d. h. ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände gegenüber einem anderen Molekül als Elektron-Donor wirkt, kann gegenüber diesem Molekül unter abweichenden experimentellen Bedingungen oder gegenüber einem dritten Molekül unter gleichen oder abweichenden experimentellen Bedingungen auch als Elektron-Akzeptor wirken.

Elekron-Akzeptor

Der Begriff Elektron-Akzeptor bezeichnet im Rahmen der vorliegenden Erfindung einen Bestandteil einer photoinduzierbar redoxaktiven Einheit bzw. einer chemisch induzierbar

redoxaktiven Einheit. Bei einem Elektron-Akzeptor handelt es sich um ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände ein Elektron von einem Elektron-Donor aufnehmen kann. Ein solcher äußerer Umstand ist z. B. die Lichtabsorption durch den Elektron-Donor oder -Akzeptor einer photoinduzierbar redoxaktiven Einheit. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der Elektron-Donor "D" an den/einen der Elektron-Akzeptor "A" ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D⁺A⁻ aus oxidiertem Donor und reduziertem Akzeptor. Ein weiterer solcher äußerer Umstand kann z. B. die Oxidation oder Reduktion des Elektron-Donors oder -Akzeptors der chemisch induzierbar redoxaktiven Einheit durch ein externes Oxidations- oder Reduktionsmittel sein, also z. B. die Übertragung eines Elektrons auf den Elektron-Donor durch ein Reduktionsmittel bzw. die Abgabe eines Elektrons durch den Elektron-Akzeptor an ein Oxidationsmittel sein. Diese Oxidations- bzw. Reduktionsmittel können sowohl externe redoxaktive Substanzen sein, d. h. sie sind nicht kovalent mit der redoxaktiven Einheit, dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbunden, stehen aber mit diesen, z. B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung, in Kontakt oder sie sind kovalent mit dem Nukleinsäure-Oligomer verbunden, wobei das Oxidationsbzw. Reduktionsmittel an einer Stelle des Nukleinsäure-Oligomers kovalent angebunden ist, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- oder Purin-Basen von der kovalenten Anbindungstelle der photoinduzierbar redoxaktiven Einheit entfernt ist, bevorzugt an dem der Modifikation mit redoxaktiver Einheit entgegengesetzten Ende des Oligonukleotids in der Nähe der leitfähigen Oberfläche. Die Fähigkeit als Elektron-Akzeptor oder -Donor zu wirken ist relativ, d. h. ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände gegenüber einem anderen Molekül als Elektron-Akzeptor wirkt, gegenüber diesem Molekül unter abweichenden experimentellen Bedingungen oder gegenüber einem dritten Molekül unter gleichen oder abweichenden experimentellen Bedingungen auch als Elektron-Donor wirken.

Contract of the Contract of th

Elektron-Donor-Molekül

Elekron-Akzeptor-

Molekül

Oxidationsmittel

entspricht einem Elektron-Donor.

entspricht einem Elektron-Akzeptor.

chemische Verbindung (chemische Substanz), die durch Aufnahme von Elektronen aus einer anderen chemischen Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) oxidiert. Oxidationsmittel sich analog zu einem Elektron-Akzeptor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht unmittelbar zur photoinduzierbar redoxaktiven Einheit bzw. chemisch induzierbar redoxaktiven Einheit gehörigen Elektron-Akzeptor verwendet. Nicht unmittelbar bedeutet in diesem Zusammenhang, daß das Oxidationsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das Oxidationsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäure-Oligomers. die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidinoder Purin-Basen von der kovalenten Anbindungstelle der (photoinduzierbar) redoxaktiven Einheit entfernt ist. Insbesondere kann die Elektrode das Oxidationsmittel darstellen.

Reduktionsmittel

chemische Verbindung (chemische Substanz), die durch Abgabe von Elektronen an eine andere chemische Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) reduziert. Ein Reduktionsmittel verhält sich analog zu einem Elektron-Donor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht unmittelbar zur photoinduzierbar redoxaktiven Einheit bzw. zur chemisch induzierbar redoxaktiven Einheit gehörigen Elektron-Donor verwendet. Nicht unmittelbar bedeutet in diesem Zusammenhang, daß das Reduktionsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das



Reduktionsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäure-Oligomers, mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidinoder Purin-Basen von der kovalenten Anbindungstelle der edoxaktiven Einheit entfernt ist. Insbesondere kann die Elektrode das Reduktionsmittel darstellen.

photoinduzierbar

photoinduzierbar bedeutet, daß eine gewisse Eigenschaft erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entfaltet wird. So entfaltet z. B. eine photoinduzierbar redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, unter bestimmten äußeren Umständen innerhalb der photoinduzierbar redoxaktiven Einheit eine Ladungstrennung durchzuführen, also z. B. den Zustand D*A-auszubilden, und an ein anderes geeignetes Oxidationsmittel Elektronen abzugeben oder von einem anderen geeigneten Reduktionsmittel Elektronen aufzunehmen, erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge. Ein weiteres Beispiel ist die photoinduzierbar reaktive Gruppe, d. h. eine Gruppe, die erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge reaktiv wird.

redoxaktiv

redoxaktiv bezeichnet die Eigenschaft einer redoxaktiven Einheit unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben oder von einem geeigneten Reduktionsmittel Elektronen aufzunehmen bzw. die Eigenschaft einer redoxaktiven Substanz unter bestimmten äußeren Umständen an einen geeigneten Elektron-Akzeptor Elektronen abzugeben oder von einem geeigneten Elektron-Donor Elektronen aufzunehmen.

freie, redoxaktive Substanz

freies, nicht kovalent mit der redoxaktiven Einheit, dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbundes, aber mit diesen, z. B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung, in Kontakt stehendes Oxidationsoder Reduktionsmittel, wobei die freie redoxaktive Substanz z. B. ein ungeladenes Molekül, eine beliebiges Salz oder ein redoxaktives Protein oder Enzym (Oxydoreductase) sein kann. Die freie redoxaktive Substanz ist dadurch gekennzeichnet, daß sie den oxidierten Donor (bzw. den

reduzierten Akzeptor) der photoinduzierbar redoxaktive Einheit re-reduzieren (bzw. re-oxidieren) kann bzw. daß die freie, redoxaktive Substanz den Donor (bzw. den Akzeptor) der chemisch induzierbar redoxaktiven Einheit reduzieren (bzw. oxidieren) kann.

photoinduzierbar redoxaktive Einheit

Oberbegriff für eine Einheit, die ein oder mehrere Elektron-Donor-Moleküle und ein oder mehrere Elektron-Akzeptor-Moleküle enthält, wobei dieses (diese) Elektron-Donor-Molekül(e) und/oder dieses (diese) Elektron-Akzeptor-Molekül(e) in ein oder mehrere Makromoleküle eingebettet sein können. Elektron-Donor(en) und Elektron-Akzeptor(en) können untereinander durch eine oder mehrere kovalente oder ionische Bindungen, durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, durch π-π-Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation miteinander verbunden sein, wobei kovalente Verbindungen direkte oder indirekte (z. B. über einen Spacer, nicht aber über ein Nukleinsäure-Oligomer) Verbindungen sein können. Daneben können Elektron-Donor(en) und/oder Elektron-Akzeptor(en), falls sie in ein oder mehrere Makromolekül(e) eingebettet sind, mit dem (den) Makromolekül(en) durch kovalente Anbindung an das (die) Makromolekül(e), durch Einkapseln in passende molekulare Kavitäten (Bindungstaschen) des Makromoleküls (der Makromoleküle), durch ionische Bindungen, Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation Akzeptation zwischen dem(n) Makromolekül(en) und dem(n) Elektron-Donor-Molekül(en) und/oder dem(n) Elektron-Akzeptor-Molekül(en) verbunden sein. Sind mehrere Makromoleküle Bestandteil der photoinduzierbar redoxaktiven Einheit kann die Bindung der Makromoleküle untereinander ebenfalls kovalent, ionisch, durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation erfolgen. Wesentliche Merkmale der photoinduzierbar redoxaktiven Einheit sind neben der Zusammensetzung aus Elektron-Donor(en) und Elektron-

Akzeptor(en) oder aus Elektron-Donor(en) und Elektron-Akzeptor(en) und Makromolekül(en): (i) die Einheit ist in den erfindungsrelevanten Erscheinungsformen (Elektron-Donor(en) und Elektron-Akzeptor(en) im ursprünglichen bzw. oxidierten oder reduzierten Zustand) stabil und dissoziiert nicht in ihre Bestandteile, (ii) die Einheit enthält keine Nukleinsäure, (iii) die Zusammensetzung der Einheit aus Elektron-Donor(en) und Elektron-Akzeptor(en) oder aus Elektron-Donor(en) Elektron-Akzeptor(en) und und Makromolekül(en) kann - unabhängig von der Bindung zwischen den Bestandteilen - vom Fachmann erkannt werden, da sie prinzipiell auch als Einzelmoleküle vorkommen und (iv) Elektron-Donor(en) und Elektron-Akzeptor(en) der photoinduzierbar redoxaktiven Einheit wirken unter gleichen oder ähnlichen äußeren Umständen in Form von Einzelmolekülen in Lösung als Elektron-Donor(en) und Elektron-Akzeptor(en), d. h. auch bei freien gelösten Elektron-Donor(en) und Elektron-Akzeptor(en) unmittelbar oder nach Einwirkung bestimmter äußerer Umstände, entsprechend den Umständen die innerhalb der photoinduzierbar redoxaktiven Einheit zu einem Elektrontransfer führen, ein Elektron vom (von den) gelösten Elektron-Donor(en) auf den (die) gelösten Elektron-Akzeptor(en) übertragen werden. photoinduzierbar redoxaktive Einheit kann z. B. jedes beliebige photoinduzierbar redoxaktive Protein/Enzym oder jeder beliebige photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der/ein Elektron-Donor an einen der Elektron-Akzeptoren ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D*A- aus einem oxidierten Donor und einem reduzierten Akzeptor. Dieser Vorgang innerhalb der photoinduzierbar redoxaktiven Einheit wird als photoinduzierte Ladungstrennung bezeichnet. Bei entsprechend gewählten äußeren Umständen entfaltet die photoinduzierbar redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, an einen geeignetes Oxidationsmittel Elektronen abzugeben oder von einem geeigneten Reduktionsmittel Elektronen aufzunehmen. erst im

ladungsgetrennten Zustand, da das Reduktionsmittel (bzw. Oxidationsmittel) nur auf den oxidierten Donor (bzw. vom reduzierten Akzeptor) der photoinduzierbar redoxaktiven Einheit Elektronen überträgt (bzw. aufnimmt), z. B. in Gegenwart eines Reduktionsmittels, das D*, jedoch nicht D, reduzieren kann (bzw. in Gegenwart eines Oxidationsmittels das A-, jedoch nicht A, oxidieren kann). Insbesondere kann dieses Oxidations- bzw. Reduktionsmittel auch eine Elektrode sein, wobei die photoinduzierbar redoxaktive Einheit erst nach der photoinduzierten Ladungstrennung ein Elektron an eine Elektrode abgeben (bzw. von dieser aufnehmen) kann, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert (bzw. D⁺, jedoch nicht D, reduziert) wird.

redoxaktive Einheit

chemisch induzierbar entspricht in Zusammensetzung und Funktionsweise einer photoinduzierbar redoxaktive Einheit, wobei aber im Unterschied zur Funktionsweise einer photoinduzierbar redoxaktiven Einheit Photoaktivierung als äußerer Umstand zur Entfaltung der Redoxaktivität der redoxaktive Einheit ausgeschlossen ist. Die redoxaktive Einheit kann also z. B. jedes beliebige redoxaktive Protein/Enzym oder jeder beliebige redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Bei entsprechend gewählten äußeren Umständen entfaltet die redoxaktive Einheit Redoxaktivität, also ihre Eigenschaft, z. B. an ein geeignetes Oxidationsmittel Elektronen abzugeben. erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D", der nur im reduzierten Zustand "D⁻ " ein Elektron auf den Akzeptor "A" übertragen kann und das Oxidationsmittel nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einneit Elektronen aufnimmt, also in Gegenwart eines Oxidationsmittels, das A-, jedoch nicht A, oxidieren kann (sukzessive Ladungsübertragung). Insbesondere kann dieses Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann bei abweichend gewählten äußeren Umständen die redoxaktive Einheit ihre Redoxaktivität, also z. B. ihre Eigenschaft von einem geeigneten Reduktionsmittel

photoinduzierbar redoxaktives Protein/Enzym

Elektronen aufzunehmen, erst nach Übertragung eines Elektrons von einem Elektron-Akzeptor "A" auf ein Oxidationsmittel entfalten, wenn nur der oxidierte Akzeptor "A* " ein Elektron vom Donor D aufnehmen kann und das Reduktionsmittel nur auf den oxidierten Donor "D*" der redoxaktiven Einheit Elektronen übertragen kann, z. B. in Gegenwart eines Reduktionsmittels, das D*, jedoch nicht D, reduziert kann (sukzessive Ladungsübertragung). Insbesondere kann dieses Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D*, jedoch nicht D, reduziert wird.

besteht in der Regel aus sogenanntem Apoprotein, dem (den) bevorzugten Makromolekül(en) der vorliegenden Erfindung, und Cofaktoren, den Elektron-Donor(en) und Elektron-Akzeptor(en) im Sinne der vorliegenden Erfindung. Die photoinduzierte Ladungstrennung innerhalb des photoaktivierbar redoxaktiven Proteins/Enzyms wird durch Licht bestimmter oder beliebiger Wellenlänge ausgelöst. So sind zum Beispiel im photosynthetischen Reaktionszentrum (reaction center, RC) als Cofaktoren ein primärer Elektron-Donor P und mehrere verschiedene Elektron-Akzeptoren A, darunter auch Quinon-Cofaktor(en) Q, in eine Proteinmatrix eingebettet und bilden so eine "polymolekulare" Einheit (vgl. Struktur 1). Die Einbettung erfolgt in diesem Fall durch Einkapselung der Cofaktoren in passende Kavitäten, sogennante Bindungstaschen der Proteinmatrix aus mehreren Protein-Untereinheiten. Sowohl die Protein-Untereinheiten als auch die Einkapselung der Cofaktoren in die Proteinmatrix ist im Fall einiger natürlich vorkommender RC durch nichtkovalente realisiert. Bindungen Bei Lichteinstrahlung geeigneter Wellenlänge gibt der primäre Donor ein Elektron an einen der Elektron-Akzeptoren ab und es bildet sich, zumindest temporär, aus den anfänglich neutralen Cofaktoren ein ladungsgetrennter RC-Zustand P*A-, insbesondere auch der Zustand P*Q".

Enzym

chemisch induzierbar entspricht in Zusammensetzung und Funktionsweise einem redoxaktives Protein/| photoinduzierbar redoxaktiven Protein/Enzym, wobei aber im Unterschied zur Funktionsweise einer photoinduzierbar

photoinduzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex

redoxaktiven Einheit Photoaktivierung als äußerer Umstand zur Entfaltung der Redoxaktivität redoxaktive der Einheit ausgeschlossen ist; das chemisch induzierbar redoxaktive Protein/Enzym besteht in der Regel aus sogenanntem Apoprotein, dem (den) bevorzugten Makromolekül(en) der vorliegenden Erfindung, und Cofaktoren, den Elektron-Donor(en) und Elektron-Akzeptor(en) im Sinne vorliegenden Erfindung. Die Eigenschaft des redoxaktiven Proteins/Enzyms zur sukzessiven Ladungsübertragung wird durch eine freie redoxaktive Substanz (Substrat) ausgelöst.

Verbindung aus einem oder mehreren Elektron-Donor Molekülen D1, D2, D3 etc. und mindestens einem oder mehreren geeigneten Elektron-Akzeptor Molekülen A1, A2, A3 etc., wobei die Elektron-Donor(en) und Elektron-Akzeptor(en) untereinander durch eine oder mehrere kovalente oder ionische Bindungen, durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, durch π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation miteinander verbunden sind. Kovalente Verbindungen in diesem Sinne können direkte oder indirekte (z. B. über einen Spacer, nicht aber über ein Nukleinsäure-Oligomer) Verbindungen sein. Wesentliche Merkmale des photoinduzierbar redoxaktiven, verknüpften, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes sind neben der Zusammensetzung aus Elektron-Donor(en) und Elektron-Akzeptor(en): (i) der Elekton-Donor-/Elektron-Akzeptor-Komplex ist in den erfindungsrelevanten Erscheinungsformen (Elektron-Donor(en) Elektron-Akzeptor(en) und ursprünglichen bzw. oxidierten oder reduzierten Zustand) stabil und dissoziiert nicht in seine Bestandteile, (ii) die Einheit enthält keine Nukleinsäure, (iii) die Zusammensetzung des wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes aus Elektron-Donor(en) und Elektron-Akzeptor(en) kann - unabhängig von der Bindung zwischen den Bestandteilen - vom Fachmann erkannt werden und (iv) Elektron-Donor(en) und Elektron-Akzeptor(en) des photoinduzierbar redoxaktiven. verknüpften, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplex



wirken unter gleichen oder ähnlichen äußeren Umständen auch in Form von Einzelmolekülen in Lösung als Elektron-Donor(en) und Elektron-Akzeptor(en), d. h. auch bei freien gelösten Elektron-Donor(en) und Elektron-Akzeptor(en) kann unmittelbar oder nach Einwirkung bestimmter äußerer Umstände, entsprechend den Umständen die innerhalb der photoinduzierbar redoxaktiven Einheit zu einem Elektrontransfer führen, ein Elektron vom (von den) gelösten Elektron-Donor(en) auf den (die) Elektron-Akzeptor(en) gelösten übertragen werden. photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex entspricht in seiner erfindungsrelevanten Funktionsweise einem photoinduzierbar redoxaktiven Protein/Enzym, d. h. auch hier kommt es durch Lichteinstrahlung geeigneter Wellenlänge zur photoinduzierten Ladungstrennung und es wird, zumindest temporär, ein ladungsgetrennter Zustand D⁺A⁻ gebildet (wobei D für ein beliebiges D1, D2, D3 etc. und A für ein beliebiges A1, A2, A3 etc. steht). Im Ausdruck "photoinduzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/ Elektron-Akzeptor-Komplex* steht der Begriff "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn der Donor mit dem Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden ist.

chemisch induzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex entspricht in Zusammensetzung und Funktionsweise einem photoinduzierbar redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex. wobei aber im Unterschied zur Funktionsweise eines photoinduzierbar redoxaktiven. verknüpften. wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes Photoaktivierung als äußerer Umstand zur Entfaltung der Redoxaktivität der redoxaktiven Einheit ausgeschlossen ist. Bei entsprechend gewählten äußeren Umständen entfaltet die redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, B. an ein geeignetes Oxidationsmittel Elektronen abzugeben, erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D", der nur im reduzierten Zustand "D" " ein Elektron auf den Akzeptor "A"

übertragen kann und das Oxidationsmittel nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einheit Elektronen aufnimmt, also in Gegenwart eines Oxidationsmittels das A-, iedoch nicht Α. oxidieren kann (sukzessive Ladungsübertragung). Insbesondere kann dieses Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann bei abweichend gewählten äußeren Umständen die redoxaktive Einheit ihre Redoxaktivität, also z. B. ihre Eigenschaft von einem geeigneten Reduktionsmittel Elektronen aufzunehmen, erst nach Übertragung eines Elektrons von einem Elektron-Akzeptor "A" auf ein Oxidationsmittel entfalten, wenn nur der oxidierte Akzeptor "A* " ein Elektron vom Donor D aufnehmen kann und das Reduktionsmittel nur auf den oxidierten Donor "D*" der redoxaktiven Einheit Elektronen übertragen kann, z. B. in Gegenwart eines Reduktionsmittels, das D⁺, jedoch nicht D, reduzieren kann (sukzessive Ladungsübertragung). Insbesondere kann dieses Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D+, jedoch nicht D, reduziert wird. Im Ausdruck "redoxaktiver. verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex" steht der Begriff "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn der Donor mit dem Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden ist.

Reaktionszentrum. Beispiel eines photoinduzierbar redoxaktiven Proteins/Enzyms. Bei dem Protein/Enzym handelt es sich um einen sogenannten Pigment/Protein-Komplex der aus Apoprotein mit mehreren Proteinuntereinheiten und mehreren Cofaktoren (im Beispiel RC sogenannte Pigmente) handelt. ln solchen Pigment/Protein-Komplexen spielen sich die ersten Schritte der lichtgetriebenen Ladungstrennung der bakteriellen oder pflanzlichen Photosynthese ab. Das RC der Photosynthese betreibenden Bakterien des Stammes Rhodobacter sphaeroides z. B. (vgl. Struktur 1) besteht aus drei Protein-

RC

Untereinheiten und acht Cofaktoren (Pigmenten). Die Cofaktoren, ein Bakteriochlorophyll-Dimer zwei Bakteriochlorophyll-Monomere B_{A} В_в, und zwei Bakteriopheophytin-Monomere H_A und H_B und zwei Ubichinon-50 (UQ) Moleküle Q_A und Q_B, sind in den jeweiligen Protein-Bindungstaschen (also der P-, B_Aetc. Bindungstasche) lokalisiert.

Q_A-Protein-Bindungstasche Proteinbindungstasche bzw. Proteinumgebung, in der sich der Chinon-Cofaktor Q_A befindet. In RC von z.B. Rhodobacter sphaeroides ist der Chinon-Cofaktor Q_A ein Ubichinon-50 (vgl. Struktur 1).

Q_A-Bindungstasche

Q_A-Protein-Bindungstasche

Chemische Substanzen/Gruppen

ZnBChl Zn-Bakteriochlorophyll (Formel 11 mit M = Zn)

Q allgemein für Chinon (engl. Quinone), im Beispiel 3 und den sich

darauf beziehenden Textpassagen ist Q ein modifiziertes

Anthrachinon oder Pyrrollochinolinochinon (PQQ).

UQ Ubichinon-50, RC-Cofaktor und temporärer Elektron-Akzeptor

z. B. im RC der Photosynthese betreibenden Bakterien aus z.B. Rhodobacter sphaeroides oder Rhodopseudomonas

viridis.

 $(\text{cyt } \text{c}_2)^{2+}$ | reduzierte Form des Cytochrom c_2 , ein frei bewegliches Häm-

Protein, das in der bakteriellen Photosynthese in *Rhodobacter* sphaeroides den oxidierten primären Donor P⁺ zu P reduziert;

Beispiel einer redoxaktiven Substanz.

PQQ Pyrrolo-Chinolino-Chinon, entspricht: 4,5-Dihydro-4,5-dioxo-

1H-pyrrolo-[2,3-f]-chinolin-2,7,9-tricarboxylsäure)

EDTA Ethylendiamin-Tetraacetat (Natriumsalz)

sulfo-NHS N-Hydroxysulfosuccinimid

EDC (3-Dimethylaminopropyl)-carbodiimid

HEPES N-[2-Hydroxyethyl]piperazin-N'-[2-ethansulfonsäure]

Tris Tris-(hydroxymethyl)-aminomethan



Alkyl

Der Begriff "Alkyl" bezeichnet eine gesättigte Kohlenwasserstoffgruppe, die geradkettig oder verzweigt ist (z.B. Ethyl, 2,5-Dimethylhexyl oder Isopropyl etc.). Wenn "Alkyl" benutzt wird, um auf einen Linker oder Spacer zu verweisen, bezeichnet der Begriff eine Gruppe mit zwei verfügbaren Valenzen für die kovalente Verknüpfung (z. B. -CH₂CH₂-, -CH,C(CH₃),CH,CH,C(CH₃),CH,oder -CH₂CH₂CH₃etc.). Bevorzugte Alkylgruppen als Substituenten oder Seitenketten R sind solche der Kettenlänge 1 - 30 (längste durchgehende Kette von kovalent aneinander gebundenen Atomen). Bevorzugte Alkylgruppen als Linker oder Spacer sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge hier die kürzeste durchgehende Verbindung zwischen den durch den Linker oder Spacer verbundenen Strukturen, also zwischen den zwei Molekülen bzw. zwischen einem Oberflächenatom, Oberflächenmolekül oder einer Oberflächenmolekülgruppe und einem anderen Molekül, darstellt.

Alkenyl

Alkylgruppen, bei denen eine oder mehrere der C-C Einfachbindungen durch C=C Doppelbindungen ersetzt sind.

Alkinyl

Alkyl- oder Alkenylgruppen, bei denen eine oder mehrere der C-C Einfach- oder C=C Doppelbindungen durch C=C Dreifachbindungen ersetzt sind.

Hetero-Alkyl

Alkylgruppen, bei denen eine oder mehrere der C-H Bindungen oder C-C Einfachbindungen durch C-N, C=N, C-P, C=P, C-O, C=O, C-S oder C=S Bindungen ersetzt sind.

Hetero-Alkenyl

Alkenylgruppen, bei denen eine oder mehrere C-H Bindungen, C-C Einfach- oder C=C Doppelbindungen durch C-N, C=N, C-P, C=P, C-O, C=O, C-S oder C=S Bindungen ersetzt sind.

Hetero-Alkinyl

Alkinylgruppen, bei denen eine oder mehrere der C-H Bindungen, C-C Einfach-, C=C Doppel- oder C≡C Dreifachbindung durch C-N, C=N, C-P, C=P, C-O, C=O, C-S oder C=S Bindungen ersetzt sind.

Linker

molekulare Verbindung zwischen zwei Molekülen bzw. zwischen einem Oberflächenatom, Oberflächenmolekül oder einer Oberflächenmolekülgruppe und einem anderen Molekül.

In der Regel sind Linker als Alkyl-, Alkenyl-, Alkinyl-, Hetero-Alkyl-, Hetero-Alkenyl- oder Heteroalkinylkette käuflich zu erwerben, wobei die Kette an zwei Stellen mit (gleichen oder verschiedenen) reaktiven Gruppen derivatisiert ist. Diese Gruppen bilden in einfachen/bekannten chemischen Reaktionen mit den entsprechenden Reaktionspartner eine kovalente chemische Bindung aus. Die reaktiven Gruppen können auch photoaktivierbar sein, d. h. die reaktiven Gruppen werden erst durch Licht bestimmter oder beliebiger Wellenlänge aktiviert. Bevorzugte Linker sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge hier die kürzeste durchgehende Verbindung zwischen den zu verbindenden Strukturen, also zwischen den zwei Molekülen bzw. zwischen einem Oberflächenatom, Oberflächenmolekül oder einer Oberflächenmolekülgruppe und einem anderen Molekül, dar. e.llt.

Spacer

Linker, der über die reaktiven Gruppen an eine oder beide der zu verbindenden Strukturen (siehe Linker) kovalent angebunden ist. Bevorzugte Spacer sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge die kürzeste durchgehende Verbindung zwischen den zu verbindenden Strukturen darstellt.

(n x HS-Spacer)oligo Nukleinsäure-Oligomer, an das n Thiolfunktionen über jeweils einen Spacer angebunden sind, wobei die Spacer jeweils eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Thiolfunktion und Nukleinsäure-Oligomer) aufweisen können, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14. Diese Spacer können verschiedene natürlich am wiederum an Nukleinsäure-Oligomer vorhandene oder an diesem durch Modifikation angebrachte reaktive Gruppen gebunden sein und "n" ist eine beliebige ganze Zahl, insbesondere eine Zahl zwischen 1 und 20.

(n x R-S-S-Spacer)oligo

Nukleinsäure-Oligomer, an das n Disulfidfunktionen über jeweils einen Spacer angebunden sind, wobei ein beliebiger Rest R die Disulfidfunktion absättigt. Der Spacer zur Anbindung der Disulfidfunktion an das Nukleinsäure-Oligomer

kann jeweils eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Disulfidfunktion und Nukleinsäure-Oligomer) aufweisen, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14. Diese Spacer können wiederum kann an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diesem durch Modifikation angebrachte reaktive Gruppen gebunden sein. Der Platzhalter n ist eine beliebige ganze Zahl, insbesondere eine Zahl zwischen 1 und 20.

oligo-Spacer-S-S-Spacer-oligo zwei gleiche oder verschiedene Nukleinsäure-Oligomere, die über eine Disulfid-Brücke miteinander verbunden sind, wobei die Disulfidbrücke über zwei beliebige Spacer an die Nukleinsäure-Oligomere angebunden ist und die beiden Spacer eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Disulfidbrücke und dem jeweiligen Nukleinsäure-Oligomer) aufweisen können, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14 und diese Spacer wiederum an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diese durch Modifikation angebrachte reaktive Gruppen gebunden sein können.

Modifizierte Oberflächen/Elektroden

Mica

Muskovit-Plättchen, Trägermaterial zum Aufbringen dünner Schichten.

Au-S-(CH₂)₂-ss-oligo-Spacer-UQ(RC) Gold-Film auf Mica mit kovalent aufgebrachter Monolayer aus derivatisiertem 12Bp Einzelstrang DNA-Oligonukleotid (Sequenz: TAGTCGGAAGCA). Hierbei ist die endständige Phosphatgruppe des Oligonukleotids am 3' Ende mit (HO-(CH₂)₂-S)₂ zum P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert, wobei die S-S Bindung homolytisch gespalten wird und je eine Au-S-R Bindung bewirkt. Die endständige Base Thymin am 5'- Ende des Oligonukleotids ist am C-5 Kohlenstoff mit -CH=CH-CO-NH-CH₂-CH₂-NH₂ modifiziert, wobei dieser Rest wiederum über seine freie Aminogruppe durch Amidbildung mit der Carbonsäuregruppe des modifizierten Ubichinon-50 verbunden ist. Anschließend wird das UQ mit dem restlichen RC rekonstituiert.

Au-S-(CH₂)₂-ds-oligo-

| Au-S-(CH2)2-ss-oligo-Spacer-UQ(RC) hybridisiert mit dem zu ss-

Spacer-UQ(RC) oligo (Sequenz: TAGTCGGAAGCA) komplementären Oligonukleotid.

Au-S-(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl

identisch zu Au-S-(CH₂)₂-ss-oligo-Spacer-UQ(RC) mit der Ausnahme, daß, statt des über UQ angebundenen RCs, Q-ZnBChl als photoinduzierbar redoxaktive Einheit angebunden ist.

Au-S-(CH₂)₂-ds-oligo-Spacer-Q-ZnBChl Au-S-(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl hybridisiert mit dem zu ss-oligo (Sequenz: TAGTCGGAAGCA) komplementären Oligonukleotid.

Elektrochemie

Amperometrie

Ε

Elektrodenpotential, das an der Arbeitselektrode anliegt.

E_{ox} Potential beim Strom-Maximum der Oxidation einer reversiblen Elektrooxidation oder -reduktion.

Stromdichte (Strom pro cm² Elektrodenoberfläche)

Cyclovoltametrie Aufzeichnung einer Strom/Spannungskurve Hierbei w

Aufzeichnung einer Strom/Spannungskurve. Hierbei wird das Potential einer stationären Arbeitselektrode zeitabhängig linear verändert, ausgehend von einem Potential, bei dem keine Elektrooxidation oder -reduktion stattfindet bis zu einem Potential, bei dem eine gelöste oder an die Elektrode adsorbierte Spezies oxidiert oder reduziert wird (also Strom fließt). Nach Durchlaufen des Oxidations- bzw. Reduktionsvorgangs, der in der Strom/Spannungskurve einen zunächst ansteigenden Strom und nach Erreichen eines Maximums einen allmählich abfallenden Strom erzeugt, wird die Richtung des Potentialvorschubs umgekehrt. Im Rücklauf wird dann das Verhalten der Produkte der Elektropyidation oder reduktion guffagseichent

Elektrooxidation oder -reduktion aufgezeichnet.

Aufzeichnung einer Strom/Zeitkurve. Hierbei wird das Potential einer stationären Arbeitselektrode z. B. durch einen Potentialsprung auf ein Potential gesetzt, bei dem die Elektrooxidation oder -reduktion einer gelösten oder adsorbierten Spezies stattfindet und der fließende Strom wird in Abhängigkeit von der Zeit aufgezeichnet

von der Zeit aufgezeichnet.

Die vorliegende Erfindung betrifft ein Nukleinsäure-Oligomer, das durch chemische Bindung einer redoxaktiven Einheit modifiziert ist. Die redoxaktive Einheit ist entweder eine photoinduzierbar redoxaktive Einheit oder eine chemisch induzierbar redoxaktive Einheit. Die photoinduzierbar redoxaktive Einheit kann nach

photoinduzierter Abgabe eines Elektrons an ein externes Oxidationsmittels, z. B. einer Elektrode, oder Aufnahme eines Elektrons von einem externen Reduktionsmittel, z. B. einer Elektrode, durch eine freie redoxaktive Substanz rereduziert bzw. re-oxidiert, also in seinen ursprünglichen Zustand zurückversetzt werden. Die chemisch induziert redoxaktive Einheit kann nach Abgabe eines Elektrons an ein externes Oxidationsmittels von einem externen Reduktionsmittel, z. B. einer Elektrode, reduziert oder nach Aufnahme eines Elektrons von einem externen Reduktionsmittel durch ein externes Oxidationsmittel, z. B. einer Elektrode, oxidiert werden.

Als Nukleinsäure-Oligomer wird im Rahmen der vorliegenden Erfindung eine Verbindung aus wenigstens zwei kovalent verbundenen Nukleotiden oder aus wenigstens zwei kovalent verbundenen Pyrimidin- (z. B. Cytosin, Thymin oder Uracil) oder Purin-Basen (z. B. Adenin oder Guanin), bevorzugt ein DNA-, RNA- oder PNA-Fragment, verwendet. In der vorliegenden Erfindung bezieht sich der Begriff Nukleinsäure auf ein beliebiges "Rückgrat" der kovalent verbundenen Pyrimidin- oder Purin-Basen, wie z. B. auf das Zucker-Phosphat Rückgrat der DNA, cDNA oder RNA, auf ein Peptid-Rückgrat der PNA oder auf analoge Rückgrat-Strukturen, wie z. B. ein Thio-Phosphat-, ein Dithio-Phosphat- oder ein Phosphoramid-Rückgrat. Wesentliches Merkmal einer Nukleinsäure im Sinne der vorliegenden Erfindung ist, daß sie natürlich vorkommende cDNA oder RNA sequenzspezifisch binden kann. Alternativ zu dem Begriff "Nukleinsäure-Oligomer" werden die Begriffe "(Sonden-) Oligonukleotid", "Nukleinsäure" oder "Oligomer" verwendet.

Der Begriff "Elektron-Akzeptor" bzw. "Elektron-Akzeptor-Molekül" und der Begriff "Elektron-Donor" bzw. "Elektron-Donor-Molekül" bezeichnet im Rahmen der vorliegenden Erfindung einen Bestandteil einer redoxaktiven Einheit.

Unter einer "redoxaktiven Einheit" wird im Rahmen der vorliegenden Erfindung jede Einheit verstanden, die einen oder mehrere Elektron-Donor-Moleküle und einen oder mehrere Elektron-Akzeptor-Moleküle enthält. Die Elektron-Donor-Molekül(e) oder - Molekülteil(e) und Elektron-Akzeptor-Molekül(e) oder - Molekülteil(e) dieser redoxaktiven Einheit können untereinander durch eine oder mehrere kovalente oder ionische Bindungen, durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, durch π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation miteinander verbunden sein, wobei kovalente Bindungen direkte oder indirekte (z. B. über einen Spacer, nicht aber über ein Nukleinsäure-Oligomer) Bindungen sein können. Außerdem können die Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-Molekül(e) in ein oder mehrere Makromolekül(e)

eingebunden sein, wobei diese Einbindung durch Einkapseln in passende molekulare Kavitäten (Bindungstaschen) des Makromoleküls (der Makromoleküle), durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation zwischen dem(n) Makromolekül(en) und dem(n) Elektron-Donor-Molekül(en) und/oder dem(n) Elektron-Akzeptor-Molekül(en) erfolgt. In diesem Fall bilden also die Makromolekül(e) und die Elektron-Donor-Molekül(e) und die Elektron-Akzeptor-Molekül(e) die redoxaktive Einheit. Sind mehrere Makromoleküle Bestandteil der redoxaktiven Einheit kann die Bindung der Makromoleküle untereinander ebenfalls kovalent, ionisch, durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation erfolgen.

Die angesprochenen Donor- und Akzeptor-Moleküle bilden erfindungsgemäß eine redoxaktive Einheit, d. h. sie sind direkt oder über weitere Molekülteile aneinander gebunden. Einzige erfindungsgemäße Einschränkung der die Bestandteile der redoxaktiven Einheit verbindenden Moleküle oder Molekülteile ist der Ausschluß von Nukleinsäure-Oligomeren. Gemäß der vorliegenden Erfindung ist die redoxaktive Einheit als eine komplette Einheit an das Sonden-Oligonukleotid gebunden, wobei natürlich mehrere chemische Bindungen zwischen Oligonukleotid und der redoxaktiven Einheit ausgebildet werden können. Durch den Ausschluß von Nukleinsäure-Oligomeren als die die Bestandteile der redoxaktiven Einheit verbindenden Moleküle oder Molekülteile soll verdeutlicht werden, daß nicht einzelne Teile der redoxaktiven Einheit an verschiedenen Stellen des Sonden-Oligonukleotids angebunden sind. Das Sonden-Oligonukleotid stellt also explizit nicht die Verbindung zwischen den Elektron-Donor-Molekül(en) oder -Molekülteil(en) und den Elektron-Akzeptor-Molekül(en) oder -Molekülteil(en) der redoxaktiven Einheit dar.

Die redoxaktive Einheit ist entweder eine photoinduzierbar redoxaktive Einheit oder eine chemisch induzierbar redoxaktive Einheit.

"Photoinduzierbar" heißt im Rahmen der vorliegenden Erfindung, daß die Redoxaktivität der photoinduzierbar redoxaktiven Einheit, also deren Eigenschaft unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben oder von einem geeigneten Reduktionsmittel Elektronen aufzunehmen, erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entfaltet wird. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der Elektron-Donor "D" an einen der Elektron-Akzeptoren "A" ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand

D⁺A⁻ aus oxidiertem Donor und reduziertem Akzeptor. Dieser Vorgang innerhalb der photoinduzierbar redoxaktiven Einheit wird als photoinduzierte Ladungstrennung bezeichnet. Bei entsprechend gewählten äußeren Umständen entfaltet die photoinduzierbar redoxaktive Einheit ihre Redoxaktivität erst im ladungsgetrennten Zustand, da das Reduktionsmittel (bzw. das Oxidationsmittel) nur auf den oxidierten Donor (bzw. vom reduzierten Akzeptor) der photoinduzierbar redoxaktiven Einheit Elektronen übertragen kann (bzw. aufnehmen kann), z. B. in Gegenwart eines Oxidationsmittels, das A⁻, jedoch nicht A, oxidieren kann (bzw. in Gegenwart eines Reduktionsmittels, das D⁺, jedoch nicht D, reduzieren kann).

Insbesondere kann das angesprochene Oxidations- bzw. Reduktionsmittel eine Elektrode sein, wobei die photoinduzierbar redoxaktive Einheit erst nach der photoinduzierten Ladungstrennung ein Elektron an die Elektrode abgeben (bzw. von dieser aufnehmen) kann, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem An, jedoch nicht A, oxidiert (bzw. Dn, jedoch nicht D, reduziert) wird. Daneben kann das Oxidations- bzw. Reduktionsmittel eine freie, redoxaktive Substanz sein, webei die photoinduzierbar redoxaktive Einheit erst nach der photoinduzierban Ladungstrennung ein Elektron an die freie, redoxaktive Substanz abgeben (bzw. von dieser aufnehmen) kann, z. B. wenn die freie redoxaktive Substanz An, jedoch nicht A, oxidiert (bzw. Dn, jedoch nicht D, reduziert).

"Chemisch induzierbar" heißt im Rahmen der vorliegenden Erfindung, daß die Redoxaktivität der chemisch induzierbar redoxaktiven Einheit eine redoxaktive Einheit verstanden, also deren Eigenschaft unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben (bzw. von einem geeigneten Reduktionsmittel Elektronen aufzunehmen), erst nach Reduktion (bzw. nach Oxidation) durch ein externes Reduktionsmittel (bzw. Oxidatinsmittel) entfaltet wird. Die chemisch induzierbar redoxaktiven Einheit entspricht in Zusammensetzung und Funktionsweise einer photoinduzierbar redoxaktive Einheit, wobei aber im Unterschied zur Funktionsweise einer photoinduzierbar redoxaktiven Einheit Photoaktivierung als äußerer Umstand zur Entfaltung der Redoxaktivität der redoxaktive Einheit ausgeschlossen ist. Bei entsprechend gewählten äußeren Umständen entfaltet die chemisch induzierbar redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, z. B. an ein geeignetes Oxidationsmittel Elektronen abzugeben, erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D": Nur im reduzierten Zustand "D" " kann der Elektron-Donor ein Elektron auf den Akzeptor "A" übertragen und das Oxidationsmittel kann nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einheit Elektronen aufnehmen. z.B. in Gegenwart eines Oxidationsmittels das A-, jedoch nicht A, oxidieren kann

(sukzessive Ladungsübertragung). Insbesondere kann das besagte Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann - bei abweichend gewählten äußeren Umständen - die chemisch induzierbar redoxaktive Einheit ihre Redoxaktivität, also z. B. ihre Eigenschaft von einem geeigneten Reduktionsmittel Elektronen aufzunehmen, erst nach Übertragung eines Elektrons von einem Elektron-Akzeptor "A" auf ein Oxidationsmittel entfalten: Nur im oxidierten Zustand "A+" kann der Elektron-Akzeptor ein Elektron vom Donor D aufnehmen und das Reduktionsmittel kann nur auf den oxidierten Donor "D+" der redoxaktiven Einheit Elektronen übertragen, z. B. in Gegenwart eines Reduktionsmittels, das D+, jedoch nicht D, reduzieren kann (sukzessive Ladungsübertragung). Insbesondere kann das besagte Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D+, jedoch nicht D, reduziert wird.

Wesentliche Merkmale der photoinduzierbar oder chemisch induzierbar redoxaktiven Einheit sind neben der Zusammensetzung aus Elektron-Donor(en) und Elektron-Akzeptor(en) oder aus Elektron-Donor(en) und Elektron-Akzeptor(en) und Makromolekül(en): (i) die Einheit ist in den erfindungsrelevanten Erscheinungsformen (Elektron-Donor(en) und Elektron-Akzeptor(en) im ursprünglichen bzw. oxidierten oder reduzierten Zustand) stabil und dissoziiert nicht in ihre Bestandteile, (ii) die Einheit enthält keine Nukleinsäure, (iii) die Zusammensetzung der Einheit aus Elektron-Donor(en) und Elektron-Akzeptor(en) oder aus Elektron-Donor(en) und Elektron-Akzeptor(en) und Makromolekül(en) kann - unabhängig von der Bindung zwischen den Bestandteilen - vom Fachmann erkannt werden, da Elektron-Donor(en) und Akzeptor(en) prinzipiell auch als Einzelmoleküle vorkommen können und (iv) Elektron-Donor(en) und Elektron-Akzeptor(en) der redoxaktiven Einheit wirken unter gleichen oder ähnlichen äußeren Umständen wie in ihrer erfindungsrelevanten Erscheinung als Bestandteile der redoxaktiven Einheit auch als Einzelmoleküle in Lösung als Elektron-Donor(en) und Elektron-Akzeptor(en), d. h. auch bei freien gelösten Elektron-Donor(en) und Elektron-Akzeptor(en) kann unmittelbar oder nach Einwirkung bestimmter äußerer Umstände, entsprechend den Umständen die innerhalb der redoxaktiven Einheit zu einem Elektrontransfer führen, ein Elektron vom (von den) gelösten Elektron-Donor(en) auf den (die) gelösten Elektron-Akzeptor(en) übertragen werden. Wie für die Elekron-Donor(en) und Elektron-Akzeptor(en) der photoinduzierbar redoxaktiven Einheit kann ein solcher äußerer Umstand für die freien, gelösten Elekron-Donor(en) und Elektron-Akzeptor(en) die Lichtabsorption durch den (die) freien, gelösten Elektron-Donor(en) oder -Akzeptor(en) sein, wobei der (ein) Elektron-Donor "D" an den (einen) Elektron-Akzeptor "A" ein Elektron abgibt und, zumindest temporär, ein ladungsgetrennter Zustand D⁺A⁻ aus einem freien, gelösten oxidierten

Donor und einem freien gelösten, reduzierten Akzeptor gebildet wird. Ein weiterer solcher äußerer Umstand kann - wie für die Elektron-Donor(en) und Elektron-Akzeptor(en) der chemisch induzierbar redoxaktiven Einheit - die Übertragung eines Elektrons auf den freien, gelösten Elektron-Donor durch ein Reduktionsmittel bzw. die Abgabe eines Elektrons durch den freien, gelösten Elektron-Akzeptor an ein Oxidationsmittel sein.

Die photoinduzierbar redoxaktive Einheit kann z. B. jedes beliebige photoinduzierbar redoxaktive Protein/Enzym oder jeder beliebige photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Im Ausdruck "photoinduzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex" steht der Begriff "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn dieser Donor und dieser Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden sind. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der/ein Elektron-Donor an einen der Elektron-Akzeptoren ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D*A" aus einem oxidierten Donor und einem reduzierten Akzeptor. Dieser Vorgang innerhalb der photoinduzierbar redoxaktiven Einheit wird als photoinduzierte Ladungstrennung bezeichnet. Bei entsprechend gewählten äußeren Umständen entfaltet die photoinduzierbar redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, an einen geeignetes Oxidationsmittel Elektronen abzugeben oder von aeeianeten. Reduktionsmittel Elektronen aufzunehmen, ladungsgetrennten Zustand, da das Reduktionsmittel (bzw. Oxidationsmittel) nur auf den oxidierten Donor (bzw. vom reduzierten Akzeptor) der photoinduzierbar redoxaktiven Einheit Elektronen überträgt (bzw. aufnimmt), z. B. in Gegenwart eines Reduktionsmittels, das D*, jedoch nicht D, reduziert kann (bzw. in Gegenwart eines Oxidationsmittels das A-, jedoch nicht A, oxidieren kann). Insbesondere kann dieses Oxidations- bzw. Reduktionsmittel auch eine Elektrode sein, wobei die photoinduzierbar redoxaktive Einheit erst nach der photoinduzierten Ladungstrennung ein Elektron an eine Elektrode abgeben (bzw. von dieser aufnehmen), z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, iedoch nicht A, oxidiert (bzw. D*, jedoch nicht D, reduziert) wird.

Die chemisch induzierbar redoxaktive Einheit kann z. B. jedes beliebige chemisch induzierbar redoxaktive Protein/Enzym oder jeder beliebige chemisch induzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Im Ausdruck "chemisch induzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex" steht der

Begriff "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn dieser Donor und dieser Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden sind. Bei entsprechend gewählten äußeren Umständen entfaltet die chemisch induzierbar redoxaktive Einheit ihre Redoxaktivität, also deren Eigenschaft unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben (bzw. von einem geeigneten Reduktionsmittel Elektronen aufzunehmen), erst nach Reduktion (bzw. nach Oxidation) durch ein externes Reduktionsmittel (bzw. Oxidationsmittel). Erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D" kann der dann reduzierte Donor "D" " ein Elektron auf den Akzeptor "A" übertragen und das Oxidationsmittel kann nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einheit Elektronen aufnehmen. Insbesondere kann das angesprochene Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann - bei abweichend gewählten äußeren Umständen - der Elektron-Akzeptor der chemisch induzierbar redoxaktive Einheit erst nach Übertragung eines Elektrons vom Akzeptor 'A' auf ein externes Oxidationsmittel in seinem dann oxidierten Zustand "A" " ein Elektron vom Donor D aufnehmen und das Reduktionsmittel nur auf den oxidierten Donor "D" " der redoxaktiven Einheit Elektronen übertragen. Insbesondere kann das angesprochene Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D⁺, jedoch nicht D, reduziert wird.

Mit dem Begriff "Oxidationsmittel" wird im Rahmen der vorliegenden Erfindung eine chemische Verbindung (chemische Substanz) bezeichnet, die durch Aufnahme von Elektronen aus einer anderen chemischen Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) oxidiert. Das Oxidationsmittel verhält sich analog zu einem Elektron-Akzeptor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht zur redoxaktiven Einheit gehörigen Elektron-Akzeptor verwendet. "Nicht unmittelbar" bedeutet in diesem Zusammenhang, daß das Oxidationsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das Oxidationsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäure-Oligomers, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Purin-Basen von der kovalenten Anbindungstelle der redoxaktiven Einheit entfernt ist. Insbesondere kann die Elektrode das Oxidationsmittel darstellen.

Mit dem Begriff "Reduktionsmittel" wird im Rahmen der vorliegenden Erfindung eine chemische Verbindung (chemische Substanz) bezeichnet, die durch Abgabe von Elektronen an eine andere chemische Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) reduziert. Das Reduktionsmittel verhält sich analog zu einem Elektron-Donor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht unmittelbar zur redoxaktiven Einheit gehörigen Elektron-Donor verwendet. "Nicht unmittelbar" bedeutet in diesem Zusammenhang, daß das Reduktionsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das Reduktionsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäure-Oligomers, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- oder Purin-Basen von der kovalenten Anbindungstelle der redoxaktiven Einheit entfernt ist. Insbesondere kann die Elektrode das Reduktionsmittel darstellen.

Mit dem Begriff "freie redoxaktive Substanz" wird im Rahmen der vorliegenden Erfindung ein freies, nicht kovalent mit der redoxaktiven Einheit, dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbundenes, aber mit diesen, z. B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung, in Kontakt stehendes Oxidationsoder Reduktionsmittel bezeichnet, wobei die freie redoxaktive Substanz z. B. ein ungeladenes Molekül, eine beliebiges Salz oder ein redoxaktives Protein oder Enzym (Oxydoreductase) sein kann. Die freie redoxaktive Substanz ist dadurch gekennzeichnet, daß sie den oxidierten Donor (bzw. den reduzierten Akzeptor) der photoinduzierbar redoxaktive Einheit re-reduzieren (bzw. re-oxidieren) kann bzw. daß die freie, redoxaktive Substanz den Donor (bzw. den Akzeptor) der chemisch induzierbar redoxaktiven Einheit reduzieren (bzw. oxidieren) kann. Desweiteren ist die freie redoxaktive Substanz dadurch gekennzeichnet, daß sie bei einem Potential φ oxidierbar und reduzierbar ist, wobei φ der Bedingung 2,0 V $\geq \varphi \geq$ - 2,0 V genügt. Das Potential bezieht sich hierbei auf das freie redoxaktive Molekül in einem geeigneten Lösungsmittel, gemessen gegen Normalwasserstoffelektrode. Im Rahmen der vorliegenden Erfindung ist der Potentialbereich 1,7 V $\geq \phi \geq$ - 1.7 V bevorzugt, wobei der Bereich 1,4 V $\geq \varphi \geq$ - 1,2 V besonders bevorzugt ist und der Bereich 0,9 V $\geq \varphi \geq$ - 0,7 V, in dem die redoxaktiven Substanzen der Anwendungsbeispiele oxidiert (bzw. reduziert) werden, ganz besonders bevorzugt ist.

Das modifizierte Nukleinsäure-Oligomer ist direkt oder indirekt (über einen Spacer) an eine leitfähige Oberfläche gebunden. Unter dem Begriff "leitfähige Oberfläche" wird jede elektrisch leitfähige Oberfläche beliebiger Dicke verstanden, insbesondere

metallische Oberflächen, Oberflächen aus Metallegierungen oder dotierte oder nicht dotierte Halbleiteroberflächen, wobei sämtliche Halbleiter als Reinsubstanzen oder als Gemische Verwendung finden können. Die leitfähige Oberfläche kann im Rahmen der vorliegenden Erfindung alleine oder auf einem beliebigen Trägermaterial, wie z. B. Glas, aufgebracht vorliegen. Im Rahmen der vorliegenden Erfindung wird der Begriff "Elektrode" alternativ zu "leitfähige Oberfläche" gebraucht.

Unter dem Begriff "modifizierte leitfähige Oberfläche" wird eine leitfähige Oberfläche verstanden, die durch Anbindung eines mit einer redoxaktiven Einheit modifizierten Nukleinsäure-Oligomers modifiziert ist.

Gemäß eines weiteren Aspekts betrifft die vorliegende Erfindung ein Verfahren, das die elektrochemische Detektion molekularer Strukturen, insbesondere die elektrochemische Detektion von DNA-/RNA-/PNA-Fragmenten in einer Probenlösung durch sequenzspezifische Nukleinsäure-Oligomer-Hybridisierung ermöglicht. Die Detektion der Hybridisierungsereignisse durch elektrische Signale ist eine einfache und kostengünstige Methode und ermöglicht in einer batteriebetriebenen Variante den Einsatz vor Ort.

Außerdem stellt die vorliegende Erfindung ein photoadressierbares Ausleseverfahren zur Detektion molekularer Strukturen zur Verfügung, unter anderem zur Detektion von Hybridisierungsereignissen auf einem Oligomer-Chip durch z. B. elektrische Signale. Erfindungsgemäß wird unter photoadressierbarem (Oligomer-Chip-) Ausleseverfahren ein Verfahren verstanden, bei dem die Detektion molekularer Strukturen auf ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe innerhalb des Gesamtsystems (des kompletten Oligomer-Chips) begrenzt wird, indem Licht bestimmter oder beliebiger Wellenlänge zur Induktion der Redoxaktivität der photoinduzierbar redoxaktiven Einheit räumlich auf diese Test-Site (-Gruppe) fokussiert (begrenzt) wird.

Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer

Voraussetzung für das erfindungsgemäße Verfahren ist die Bindung einer photoinduzierbar redoxaktiven Einheit bzw. einer chemisch induzierbar redoxaktiven Einheit an ein Nukleinsäure-Oligomer.

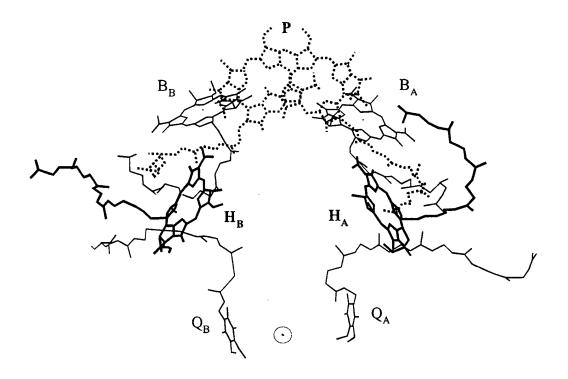
Als Beispiele einer photoinduzierbar redoxaktiven Einheit seien genannt:

(i) das photosynthetische bakterielle Reaktionszentrum (RC), wie z. B. das RC von Rhodobacter sphaeroides mit der schematischen Struktur 1, das RC anderer photosynthetischer Bakterien, wie z. B. das Reaktionszentrum von Rhodopseudomonas viridis oder von Rhodobacter capsulatus, oder ein Reaktionszentrum der Photosynthese betreibenden Pflanzen, wie z. B das Photosystem 1 oder das Photosystem 2, als Beispiele für ein photoinduzierbar redoxaktives Protein/Enzym.

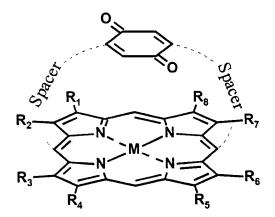
- (ii) Cyclophane, also verbrückte Porphyrin-Chinon-Systeme, der allgemeinen Struktur 2 als Beispiel für einen photoinduzierbar redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex. Die beiden Spacerverbrückten, kovalenten Verbindungen ("- - Spacer - -" in Struktur 2) zwischen dem Elektron-Akzeptor (1,4-Benzochinon in der Struktur 2) und dem Elekron-Donor (Metallo-Porphyrin in der Struktur 2) können an beliebigen Stellen des Elektron-Donors und/oder Elektron-Akzeptors angebracht sein. Neben den in der Struktur 2 gezeigten Elektron-Akzeptoren können auch Flavine der allgemeinen Formel 1, Nicotinsäureamide der allgemeinen Formel 2 oder andere Chinone, z. B. solche der allgemeinen Formeln 3 - 8 oder organische bzw. anorganische Elektron-Akzeptoren und außerdem neben den (Metallo-) Porphyrinen der allgemeinen Formel 9 andere Elektron-Donoren, wie z. B. (Metallo-)Chlorophylle der allgemeinen Formel 10 oder (Metallo-) Bakteriochlorophylle der allgemeinen Formel 11 oder andere organische bzw. anorganische Elektron-Donoren verwendet werden. Daneben können auch einfach kovalent (Spacer-)verbrückte Elektron-Donor/Elektron-Akzeptor-Komplexe wie z. B. kovalente Verbindungen einer Substanz gemäß Formel 9 und einer der Substanzen gemäß einer der Formeln 1 - 8, kovalente Verbindungen einer Substanz gemäß Formel 10 und einer der Substanzen gemäß einer der Formeln 1-8 oder kovalente Verbindungen einer Substanz gemäß Formel 11 und einer der Substanzen gemäß einer der Formeln 1 - 8 als photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elektron-Donor/Elektron-Akzeptor-Komplexe verwendet werden:
- (iii) photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplexe bei denen (einer) der Elektron-Donor(en) und/oder (einer) der Elektron-Akzeptor(en) ein Charge-Transfer-Komplex oder Übergangsmetall-Komplex ist. Beispiele für Übergangsmetall-Komplexe sind [Ru(bipy)₂(py)(im)]²⁺, beliebige andere [Ru(II)(L1)(L2)(L3)(L4)(L5)(L6)]-Komplexe, Cr(III)-, Fe(II)-, Os(II)-, oder Co(II)-Komplexe, wobei "bipy" für einen Bispyridyl-Liganden, "py" für einen Pyridyl-Liganden, "im" für einen Immidazol-Liganden und L1

bis L3 für einen beliebigen Liganden steht und auch mehr oder weniger als 6 Liganden an ein Übergangsmetall koordinieren können.

Beispiele einer chemisch induzierbar redoxaktiven Einheit sind der Cytochrom-bc Komplex oder der Cytochrom c₂ Komplex der Photosynthese betreibenden Bakterien (Komplex aus Proteinmatrix und vier eingebetteten Fe-Porphyrin Cofaktoren als Elektron-Donoren und/oder Elektron-Akzeptoren) als Beispiele für ein chemisch induzierbar redoxaktives Protein/Enzym oder, wie unter (ii) und (iii) aufgelistet, geeignet zusammengesetzte Cyclophane bzw. analoge Verbindungen als Beispiele für einen chemisch induzierbar redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex.



Struktur 1: Reaktionszentrum bestehend aus den Cofaktoren P (primärer Donor, ein Bakteriochlorophyll Dimer), B_A und B_B (Bakteriochlorophyll Monomere), H_A und H_B (Bakteriopheophytine), Q_A und Q_B (Ubichinon-50) und den Proteinuntereinheiten L, M, und H (nicht gezeigt), die die Cofaktoren einhüllen.



Struktur 2: ein Cyclophan; M = z. B. 2H, Mg, Zn, Cu, Ni, Pd, Co, Cd, Mn, Fe, Sn, Pt etc.; R_1 bis R_8 , oder Spacer sind unabhängig voneinander beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.

$$R_2$$
 R_3
 R_4
 R_5
 R_5

Formel 1

$$R_2$$
 R_3
 N^+
 R_5
 R_4

Formel 2

$$R_2$$
 R_3
 R_4
 R_5
 R_4

Formel 3

$$R_1$$
 R_2
 R_3
 R_4

Formel 4

Formel 5

$$R_2$$
 R_3
 R_4
 R_5
 R_6

Formel 6

$$R_2$$
 R_3
 R_4
 R_5

Formel 7

$$R_2$$
 R_3
 R_4
 R_5
 R_6

Formel 8

 R_1 bis R_8 sind unabhängig voneinander H oder beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.

$$R_{12}$$

$$R_{11}$$

$$R_{10}$$

R₁₂

N

N

R₄

R₅

R₁₀₁

R₈

R₈

R₇

O

Formel 9

Formel 10

$$R_{12}$$
 R_{1}
 R_{2}
 R_{3}
 R_{4}
 R_{4}
 R_{10}
 R_{10}

Formel 11

M = 2H, Mg, Zn, Cu, Ni, Pd, Co, Cd, Mn, Fe, Sn, Pt etc.; R_1 bis R_{12} sind unabhängig voneinander H oder beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.

Daneben zeichnet sich die redoxaktive Einheit erfindungsgemäß dadurch aus, daß besagte Einheit an ein ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenes Oxidationsmittel Elektronen abgibt bzw. von einen anderen ebenfalls kovalent an das Oligonukleotid angebundenen Reduktionsmittel Elektronen aufnimmt, wobei dieses Oxidations- oder Reduktionsmittel insbesondere eine elektrisch leitfähige Oberfläche (Elektrode) sein kann und die redoxaktive Einheit durch Anlegen einer äußeren Spannung an dieser Elektrode im elektrochemisch zugänglichen Potentialbereich der Elektrode elektrooxidiert/-reduziert werden kann.

Die redoxaktive Substanz zeichnet sich erfindungsgemäß dadurch aus, daß sie die photoinduzierbar redoxaktive Einheit, nach deren Elektron-Abgabe an ein anderes, von der redoxaktiven Substanz verschiedenes, kovalent an das Oligonukleotid angebundenes Oxidationsmittel re-reduzieren kann (bzw. nach deren Elektron-Aufnahme von einem anderen, von der redoxaktiven Substanz verschiedenen, kovalent an das Oligonukleotid angebundenen Reduktionsmittel re-oxidieren kann). bzw. daß die freie, redoxaktive Substanz den Donor (bzw. den Akzeptor) der chemisch induzierbar redoxaktiven Einheit reduzieren (bzw. oxidieren) kann. Erfindungsgemäß kann dazu jede redoxaktive Substanz verwendet werden, solange sie bei einem Potential ϕ , das der Bedingung 2,0 V $\geq \phi \geq$ - 2,0 V genügt, oxidierbar und reduzierbar ist und das Potential geeignet ist, besagte photoinduzierbar redoxaktive Einheit nach deren Elektron-Abgabe an ein anderes, ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenes Oxidationsmittel zu re-reduzieren (bzw. nach deren Aufnahme eines Elektrons von einen anderen, ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenen Reduktionsmittel zu re-oxidieren) oder besagte chemisch induzierbar redoxaktive Einheit zu reduzieren bzw. zu oxidieren. Das Potential bezieht sich hierbei auf die freie, unmodifizierte, redoxaktive Substanz in einem geeigneten Lösungsmittel, gemessen gegen Normalwasserstoffelektrode. Im Rahmen der vorliegenden Erfindung ist der Potentialbereich 1,7 V $\geq \phi \geq$ - 1.7 V bevorzugt, wobei der Bereich 1,4 V $\geq \phi \geq$ - 1,2 V besonders bevorzugt ist und der Bereich 0,9 V $\geq \phi \geq$ - 0,7 V, in dem die redoxaktiven Substanzen des Anwendungsbeispiels oxidiert (und rereduziert) werden, ganz besonders bevorzugt ist. Geeignet sind, neben den üblichen organischen und anorganischen redoxaktiven Molekülen wie z. B. Hexacyanoferraten, Ferrocenen, Cobaltocenen und Chinonen, vor allem die Ascorbinsäure (oder das Na⁺ Salz davon), [Ru(NH₃)₆]²⁺, Cytochrom c_2 (cyt c_2)²⁺, ein frei bewegliches eisenhaltiges Protein, das den oxidierten primären Donor P* in RC von Rhodobacter sphaeroides zu P reduziert und dabei selbst zu (cyt c₂)³⁺ oxidiert wird.

In einer bevorzugten Ausführungsform der vorliegenden Erfindung ist der photo- oder chemisch induzierbar redoxaktive, verknüpfte, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex dergestalt in ein oder mehrere Makromoleküle eingebettet, daß das Makromolekül als elektrisch isolierende Einhüllende des redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplexes wirkt, indem er die direkte Elektrooxidation/-reduktion des redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplexes an der Elektrode , z. B. bei direktem Kontakt zwischen Elektrode und redoxaktivem, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex, verhindert, die indirekte, über doppelsträngiges Nukleinsäure-Oligomer vermittelte Elektrooxidation/-reduktion Donor/Elektron-Akzeptor-Komplexes aber erlaubt. Ein solchen Makromolekül kann z. B. ein maßgeschneidertes Cyclodextrin sein, das durch seine Form eines abgeschnittenen, innen hohlen Kegels ein Cyclophan oder ähnliche Elektron-Donor/Elektron-Akzeptor-Komplex ummantelt.

Erfindungsgemäß wird eine redoxaktive Einheit an ein Nukleinsäure-Oligomer kovalent durch die Reaktion des Nukleinsäure-Oligomers mit der redoxaktiven Einheit oder Teilen davon (siehe auch Abschnitt "Wege zur Ausführung der Erfindung") gebunden. Diese Bindung kann auf vier verschiedene Arten durchgeführt werden:

a) Als reaktive Gruppe zur Bindungsbildung am Nukleinsäure-Oligomer wird eine freie Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe des Oligonukleotid-Rückgrats, insbesondere eine Gruppe an einem der beiden Enden des Oligonukleotid-Rückgrats, verwendet. Die freien, endständigen Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen weisen eine erhöhte Reaktivität auf und gehen daher leicht typische Reaktionen wie z. B. Amidbildung mit (primären oder sekundären) Aminogruppen bzw. mit Säuregruppen, Esterbildung mit (primären, sekundären oder tertiären) Alkoholen bzw. mit Säuregruppen, Thioesterbildung mit (primären, sekundären oder tertiären) Thio-Alkoholen bzw. mit Säuregruppen oder die Kondensation von Amin und Aldehyd mit anschließender Reduktion der entstandenen CH=N Bindung zur CH2-NH Bindung ein. Die zur kovalenten Anbindung der redoxaktiven Einheit nötige Kopplungsgruppe (Säure-, Amin-, Alkohol-, Thioalkohol- oder Aldehydfunktion) ist entweder natürlicherweise an der redoxaktiven Einheit vorhanden oder wird durch chemische Modifikation der redoxaktiven Einheit erhalten. Die Anbindung der redoxaktiven Einheit kann komplett oder in Teilen der Einheit mit anschließender Vervollständigung der redoxaktiven Einheit erfolgen (siehe unten).

b) Das Nukleinsäure-Oligomer ist über einen kovalent angebundenen Molekülteil (Spacer) beliebiger Zusammensetzung und Kettenlänge (längste durchgehende Kette von aneinander gebundenen Atomen), insbesondere der Kettenlänge 1 bis 14, am Oligonukleotid-Rückgrat bzw. an einer Base mit einer reaktiven Gruppe modifiziert. Die Modifikation erfolgt bevorzugt an einem der Enden des Oligonukleotid-Rückgrats bzw. an einer terminalen Base. Als Spacer kann z.B. ein Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinylsubstituent verwendet werden. Mögliche einfache Reaktionen zur Ausbildung der kovalenten Bindung zwischen redoxaktiver Einheit und des so modifizierten Nukleinsäure-Oligomers sind wie unter a) beschrieben, die Amidbildung aus Säure- und Amino-Gruppe, die Esterbildung aus Säure- und Alkohol-Gruppe, die Thioesterbildung aus Säure- und Thio-Alkohol-Gruppe oder die Kondensation von Aldehyd und Amin mit anschließender Reduktion der entstandenen CH=N Bindung zur CH₂-NH Bindung. Die Anbindung der redoxaktiven Einheit kann komplett oder in Teilen der redoxaktiven Einheit mit anschließender Vervollständigung der Einheit erfolgen (siehe unten).

- c) Bei der Synthese des Nukleinsäure-Oligomers wird eine terminale Base durch die redoxaktive Einheit ersetzt. Diese Anbindung der redoxaktiven Einheit kann komplett oder in Teilen der Einheit mit anschließender Vervollständigung der redoxaktiven Einheit erfolgen (siehe unten).
- d) Bei der Verwendung eines verknüpften (wenigstens bimolekularen) Elekton-Donor-/Elektron-Akzeptor-Komplexes als redoxaktiver Einheit wird der Elektron-Akzeptor (oder -Donor) in einer ersten kovalenten Modifikation, wie unter b) oder c) in diesem Abschnitt beschrieben, an eine oder statt einer terminalen Base an das Nukleinsäure-Oligomer gebunden und anschließend in einer zweiten kovalenten Modifikation der Elektron-Donor (oder -Akzeptor), wie unter a) in diesem Abschnitt beschrieben, am selben Ende des Nukleinsäure-Oligomer-Rückgrats an eine reaktive Gruppe des Rückgrats oder an eine reaktive Gruppe des Akzeptors (bzw. des Donors) gebunden. Bei Verwendung eines kovalent verknüpften, tri- oder höhermolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes kann, statt des Elektron-Akzeptors (oder -Donors), auch ein beliebiger Teil des Elekton-Donor-/Elektron-Akzeptor-Komplexes in der ersten kovalenten Modifikation verwendet und in einer zweiten bzw. weiteren kovalenten Modifikation(en) komplettiert werden.

Erfindungsgemäß kann die Bindung der redoxaktiven Einheit an das Nukleinsäure-Oligomer ganz oder in Teilen vor oder nach der Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche erfolgen. So kann im Falle eines redoxaktiven Proteins/Enzyms aus Apoprotein und Cofaktor(en) statt der kompletten redoxaktiven

Einheit auch nur das Apoprotein, das Apoprotein und ein Teil der Cofaktoren oder ein oder mehrere Cofaktoren angebunden sein und die redoxaktive Einheit wird durch anschließende Rekonstitution mit den noch fehlenden Teilen komplettiert. Bei der Verwendung eines verknüpften (wenigstens bimolekularen) Elekton-Donor-/Elektron-Akzeptor-Komplexes als redoxaktiver Einheit wird der Elektron-Akzeptor (oder -Donor) in einer ersten kovalenten Modifikation, wie unter b) oder c) in diesem Abschnitt beschrieben, an oder statt einer terminalen Base an das Nukleinsäure-Oligomer gebunden und anschließend in einer zweiten kovalenten Modifikation der Elektron-Donor (oder -Akzeptor), wie unter a) in diesem Abschnitt beschrieben, am selben Ende des Nukleinsäure-Oligomer-Rückgrats an eine reaktive Gruppe des Rückgrats gebunden. Bei Verwendung eines kovalent verknüpften, tri- oder höhermolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes kann, statt des Elektron-Akzeptors (oder -Donors), auch ein beliebiger Teil des Elekton-Donor-/Elektron-Akzeptor-Komplexes in der ersten kovalenten Modifikation verwendet und in der zweiten kovalenten Modifikation komplettiert werden. Diese Modifikationen können vor oder nach der Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche erfolgen.

Bei mehreren verschiedenen Nukleinsäure-Oligomer-Kombinationen (Test-Sites) auf einer gemeinsamen Oberfläche ist es vorteilhaft, die (kovalente) Anbindung der redoxaktiven Einheit an die Nukleinsäure-Oligomere durch geeignete Wahl der reaktiven Gruppe an den freien Nukleinsäure-Oligomerenden der verschiedenen Test-Sites für die gesamte Oberfläche zu vereinheitlichen, wenn die redoxaktive Einheit nach Immobilisierung des Nukleinsäure-Oligomers an der Oberfläche angebunden werden soll.

Bei Verwendung von redoxaktiven Proteinen/Enzymen als redoxaktiver Einheit kann die kovalente Anbindung des Nukleinsäure-Oligomers an eine beliebige, natürlicherweise vorhandene oder durch Modifikation angebrachte, reaktive Gruppe des Proteins erfolgen oder - in dem Falle, daß das redoxaktive Protein/Enzym aus Apoprotein und Cofaktor(en) besteht - an eine beliebige, natürlicherweise vorhandene oder durch Modifikation angebrachte, reaktive Gruppe eines (beliebigen) Cofaktors. Im Rahmen der vorliegenden Erfindung ist die kovalente Anbindung an eine beliebige, natürlicherweise vorhandene oder durch Modifikation angebrachte, reaktive Gruppe eines (beliebigen) Cofaktors des Proteins bevorzugt. Ohne an mechanistische Details gebunden sein zu wollen, ist bei mehreren Cofaktoren derjenige besonders bevorzugt, der Elektronen an ein externes, ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenes Oxidationsmittel abgeben oder von einem externen, ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenen

Reduktionsmittel aufnehmen kann (siehe auch Abschnitt "Verfahren zur amperometrischen Detektion von Nukleinsäure-Oligomer-Hybriden").

Die leitfähige Oberfläche

Unter dem Begriff "leitfähige Oberfläche" wird erfindungsgemäß jeder Träger mit einer elektrisch leitfähigen Oberfläche beliebiger Dicke verstanden, insbesondere Oberflächen aus Platin, Palladium, Gold, Cadmium, Quecksilber, Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium und Mangan.

Daneben können auch beliebige dotierte oder nicht dotierte Halbleiteroberflächen beliebiger Dicke verwendet werden. Sämtliche Halbleiter können als Reinsubstanzen oder als Gemische Verwendung finden. Als nicht einschränkend gemeinte Beispiele seien an dieser Stelle Kohlenstoff, Silizium, Germanium, α-Zinn, Cu(I)- und Ag(I)-Halogenide beliebiger Kristallstruktur genannt. Geeignet sind ebenfalls sämtliche binären Verbindungen beliebiger Zusammensetzung und beliebiger Struktur aus den Elementen der Gruppen 14 und 16, den Elementen der Gruppen 13 und 15, sowie den Elementen der Gruppen 15 und 16. Daneben können ternäre Verbindungen beliebiger Zusammensetzung und beliebiger Struktur aus den Elementen der Gruppen 11, 13 und 16 oder den Elementen der Gruppen 12, 13 und 16 verwendet werden. Die Bezeichnungen der Gruppen des Periodensystems der Elemente beziehen sich auf die IUPAC-Empfehlung von 1985.

Bindung eines Nukleinsäure-Oligomers an die leitfähige Oberfläche

Erfindungsgemäß wird ein Nukleinsäure-Oligomer direkt oder über einen Linker/Spacer mit den Oberflächenatomen oder -molekülen einer leitfähigen Oberfläche der oben beschriebenen Art verknüpft. Diese Bindung kann auf drei verschiedene Arten durchgeführt werden:

a) Die Oberfläche wird so modifiziert, daß eine reaktive Molekül-Gruppe zugänglich ist. Dies kann durch direkte Derivatisierung der Oberflächenmoleküle, z. B. durch naßchemische oder elektrochemische Oxidation/Reduktion geschehen. So kann z. B. die Oberfläche von Graphitelektroden durch Oxidation naßchemisch mit Aldehydoder Carbonsäure-Gruppen versehen werden. Elektrochemisch besteht z. B. die Möglichkeit durch Reduktion in Gegenwart von Aryl-Diazoniumsalzen das entsprechende (funktionalisierte, also mit einer reaktiven Gruppe versehene) Aryl-

Radikal oder durch Oxidation in Gegenwart von R'CO₂H das (funktionalisierte) R'-Radikal auf der Graphit-Elektrodenoberfläche anzukoppeln. Ein Beispiel der direkten Modifikation von Halbleiteroberflächen ist die Derivatisierung von Siliziumoberflächen zu reaktiven Silanolen, d. h. Silizium-Träger mit Si-OR" Gruppen an der Oberfläche, wobei R" ebenso wie R' einen beliebigen, funktionalisierten, organischen Rest darstellt (z.B. Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinylsubstituent). Alternativ kann die gesamte Oberfläche durch die kovalente Anbindung einer reaktiven Gruppe eines bifunktionalen Linkers modifiziert werden, so daß auf der Oberfläche eine monomolekulare Schicht beliebiger Moleküle entsteht, die, bevorzugt endständig, eine reaktive Gruppe enthalten. Unter dem Begriff "bifunktionaler Linker" wird jedes Molekül beliebiger Kettenlänge, insbesondere der Kettenlängen 2-14, mit zwei gleichen (homo-bifunktional) oder zwei verschiedenen (hetero-bifunktional) reaktiven Molekül-Gruppen verstanden.

Sollen mehrere verschiedene Test-Sites auf der Oberfläche durch Ausnutzen der Methodik der Photolithographie gebildet werden, so ist mindestens eine der reaktiven Gruppen des homo- oder hetereo-bifunktionalen Linkers eine photoinduzierbar reaktive Gruppe, d. h. eine erst durch Lichteinstrahlung bestimmter oder beliebiger Wellenlänge reaktiv werdende Gruppe. Dieser Linker wird so aufgebracht, daß die/eine photoaktivierbare reaktive Gruppe nach der kovalenten Anbindung des Linkers auf der Oberfläche zur Verfügung steht. An die so modifizierte Oberfläche werden die Nukleinsäure-Oligomere kovalent angebunden, wobei diese selbst über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, mit einer reaktiven Gruppe modifiziert sind, bevorzugt in der Nähe eines Endes des Nukleinsäure-Oligomers. Bei der reaktiven Gruppe des Oligonukleotids handelt es sich um Gruppen, die direkt (oder indirekt) mit der modifizierten Oberfläche unter Ausbildung einer kovalenten Bindung reagieren. Daneben kann an die Nukleinsäure-Oligomere in der Nähe ihres zweiten Endes eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die redoxaktive Einheit (komplett oder Bestandteile davon), alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Nukleinsäure-Oligomers angebunden sein.

b) Das Nukleinsäure-Oligomer, das auf die leitfähige Oberfläche aufgebracht werden soll, ist über einen kovalent angebundenen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, mit einer oder mehreren reaktiven Gruppen modifiziert, wobei sich die reaktive Gruppen bevorzugt in der

Nähe eines Endes des Nukleinsäure-Oligomers befindet. Bei den reaktiven Gruppen handelt es sich um Gruppen, die direkt mit der unmodifizierten Oberfläche reagieren können. Beispiele hierfür sind: (i) Thiol- (HS-) oder Disulfid- (S-S-) derivatisierte Oligonukleotide der allgemeinen Formel HS-Spacer-oligo bzw. R-S-S-SpacerNukleinsäure-Oligomere der allgemeinen Formel (n x HS-Spacer)-oligo, (n x R-S-S-Spacer)-oligo oder oligo-Spacer-S-S-Spacer-oligo, die mit einer Goldoberfläche unter Ausbildung einer Gold-Schwefelbindungvon Gold-Schwefelbindungen reagieren oder (ii) Amine, die sich durch Chemi- oder Physikesorption an Platin- oder Silizium-Oberflächen anlagern. Daneben kann an die OligonukleotidNukleinsäure-Oligomere in der Nähe ihres zweiten Endes (5'- oder 3'-Ende) die redexaktive Substanz oder eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. <u>Desweiteren kann die photinduzierbar</u> redoxaktive Einheit (komplett oder Bestandteile davon) alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Oligonukleotids angebunden sein. Insbesondere Nukleinsäure-Oligomere die mit mehreren Spacer-verbrückten Thiol oder Disulfidbrücken modifiziert sind ((n x HS-Spacer)-oligo bzw. (n x R-S-S-Spacer)oligo) haben den Vorteil, daß solche Nukleinsäure-Oligomere unter einem bestimmten Anstellwinkel gegen die leitfähige Oberfläche (Winkel zwischen der Oberflächennormalen und der Helixachse eines doppelsträngigen helikalen Nukleinsäure-Oligomers bzw. zwischen der Oberflächennormalen und der Achse senkrecht zu den Basenpaaren eines doppelsträngigen nicht-helikalen Nukleinsäure-Oligomers) aufgebracht werden können, wenn die die Thiol- bzw. Disulfid-Funktionen an das Nukleinsäure-Oligomer anbindenden Spacer, von einem Ende der Nukleinsäure her betrachtet, eine zunehmende bzw. abnehmende Kettenlänge besitzen.

Gruppe am Sonden-Nukleinsäure-Oligomer werden die reaktive Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen des Oligonukleotid-Rückgrass, insbesondere endständige Gruppen, verwendet. Die Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen weisen eine erhöhte Reaktivität auf und gehen daher leicht typische Reaktionen wie z. B. Amidbildung mit (primären oder sekundären) Amino- bzw. Säuregruppen, sekundären oder tertiären) Alkoholen bzw. mit (primären, Esterbilduna Säuregruppen. Thioesterbildung mit (primären, sekundären oder tertiären) Thio-Alkoholen bzw. Säuregruppen oder die Kondensation von Amin und Aldehyd mit anschließender Reduktion der entstandenen CH=N Bindung zur CH2-NH Bindung ein. Die nötige Kopplungs-Gruppe zur kovalenten Anbindung an die Phosphorsäure-,

Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe ist in diesem Fall ein Teil der Oberflächenderivatisierung mit einer (monomolekularen) Schicht beliebiger Moleküllänge, wie unter a) in diesem Abschnitt beschrieben, oder die Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe kann direkt mit der unmodifizierten Oberfläche reagieren, wie unter b) in diesem Abschnitt beschrieben. Daneben kann an die Oligonukleotide in der Nähe ihres zweiten Endes eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die redoxaktive Einheit (komplett oder Bestandteile davon), alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Nukleinsäure-Oligomers angebunden sein.

Die Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche kann vor oder nach der Anbindung der redoxaktive Einheit an das Nukleinsäure-Oligomer erfolgen. Im Falle eines redoxaktiven Proteins/Enzyms aus Apoprotein und Cofaktor(en) kann statt der kompletten redoxaktiven Einheit auch nur das Apoprotein, das Apoprotein mit einem Teil der Cofaktoren oder ein oder mehrere der Cofaktor angebunden sein und die redoxaktive Einheit wird durch anschließende Rekonstitution mit den noch fehlenden Teilen komplettiert. Bei der Verwendung eines verknüpften (wenigstens bimolekularen) Elekton-Donor-/Elektron-Akzeptor-Komplexes als redoxaktive Einheit kann der Elektron-Akzeptor (bzw. -Donor), wie unter b) oder c) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben, an eine oder statt einer terminalen Base an das Nukleinsäure-Oligomer gebunden sein und der Elektron-Donor (bzw. -Akzeptor) durch anschließende kovalente Anbindung an eine reaktive Gruppe des Elektron-Akzeptors (oder -Donors) angebunden werden oder, wie unter a) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben, durch anschließende Anbindung an eine terminale reaktive Gruppe des Nukleinsäure-Oligomer-Rückgrats am selben Ende (siehe auch den Abschnitt "Wege zur Ausführung der Erfindung"). Alternativ kann die Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche vor oder nach Anbinden des mit einer reaktiven Gruppe versehenen Spacers zur Bindung der redoxaktiven Einheit erfolgen. Die Bindung des bereits modifizierten Nukleinsäure-Oligomers an die leitfähige Oberfläche, d. h. die Bindung an die Oberfläche nach der Anbindung der redoxaktiven Einheit an das Nukleinsäure-Oligomer bzw. nach der Anbindung von Teilen der redoxaktiven Einheit oder nach Anbinden des mit einer reaktiven Gruppe versehenen Spacers zur Bindung der redoxaktiven Einheit, erfolgt ebenfalls wie unter a) bis c) in diesem Abschnitt beschrieben.

Bei der Herstellung der Test-Sites muß bei der Anbindung der Einzelstrang-Nukleinsäure-Oligomere an die Oberfläche darauf geachtet werden, daß zwischen den einzelnen Nukleinsäure-Oligomeren ein genügend großer Abstand verbleibt, um zum einen den für eine Hybridisierung mit dem Target-Nukleinsäure-Oligomer nötigen Freiraum und zum anderen den für die Anbindung der redoxaktiven Einheit nötigen Freiraum zur Verfügung zu stellen. Dazu bieten sich insbesondere drei verschiedene Vorgehensweisen (und Kombinationen daraus) an:

- 1.) Herstellung einer modifizierten Oberfläche durch Anbindung eines hybridisierten Nukleinsäure-Oligomers, also eine Oberflächen-Derivatisierung mit hybridisiertem Sonden-Nukleinsäure-Oligomer statt mit Einzelstrang-Sonden-Oligonukleotid. Der zur Hybridisierung verwendete Nukleinsäure-Oligomer-Strang ist unmodifiziert (die Oberflächenanbindung wird durchgeführt wie unter a) c) in diesem Abschnitt beschrieben). Anschließend wird der hybridisierte Nukleinsäure-Oligomer-Doppelstrang thermisch dehybridisiert, wodurch eine mit Einzelstrang- Nukleinsäure-Oligomer modifizierte Oberfläche mit größerem Abstand zwischen den Sonden-Nukleinsäure-Oligomeren hergestellt wird.
- 2.) Herstellung einer modifizierten Oberfläche durch Anbindung eines Einzelstrangoder Doppelstrang-Nukleinsäure-Oligomers, wobei während der Oberflächen-Derivatisierung ein geeigneter monofunktionaler Linker zugesetzt wird, der neben dem Einzelstrang- oder Doppelstrang-Nukleinsäure-Oligomer ebenfalls an die Oberfläche gebunden wird (die Oberflächenanbindung wird durchgeführt wie unter a) - c) in diesem Abschnitt beschrieben). Erfindungsgemäß hat der monofunktionale Linker eine Kettenlänge, die der Kettenlänge des Spacers zwischen der Oberfläche und dem Nukleinsäure-Oligomer identisch ist oder um maximal vier Kettenatome abweicht. Bei der Verwendung von Doppelstrang-Nukleinsäure-Oligomer zur Obertlächen-Derivatisierung wird der Nukleinsäure-Oligomer-Doppelstrang nach der gemeinsamen Anbindung des Doppelstrang- Nukleinsäure-Oligomers und des Linkers an die Oberfläche thermisch dehybridisiert. Durch die gleichzeitige Anbindung eines Linkers an die Oberfläche wird der Abstand zwischen den ebenfalls an die Oberfläche gebundenen Einzel- oder Doppelstrang-Nukleinsäure-Oligomeren vergrößert. Im Falle der Verwendung von Doppelstrang-Nukleinsäure-Oligomer wird dieser Effekt durch die anschließende thermische Dehybridisierung noch verstärkt.
- 3.) Herstellung einer modifizierten Oberfläche durch Anbindung eines Einzelstrangoder Doppelstrang-Oligonukleotids, an das die redoxaktive Einheit bereits angebunden ist, wobei die redoxaktive Einheit einen Durchmesser von größer als 30

Å aufweist. Bei der Verwendung von Doppelstrang-Oligonukleotid wird der Oligonukleotid-Doppelstrang nach der Anbindung des Doppelstrang-Oligonukleotids an die Oberfläche thermisch dehybridisiert.

Im Bezug auf die einzelnen Schritte zur "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" als auch zur "Bindung eines Oligonukleotids an die leitfähige Oberfläche" sei darauf verwiesen, daß im Abschnitt "Wege zur Ausführung der Erfindung" die verschiedenen Kombinationsmöglichkeiten der einzelnen Schritte, die zum selben Endergebnis führen, an einem Beispiel demonstriert sind (Figur 2).

Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybriden

Vorteilhafterweise werden gemäß dem Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybriden mehrere Sonden-Nukleinsäure-Oligomere unterschiedlicher Sequenz, idealerweise alle nötigen Kombinationen Nukleinsäure-Oligomers, auf einem Oligomer (DNA) -Chip aufgebracht, um die Sequenz eines beliebigen Target-Nukleinsäure-Oligomers oder einer (fragmentierten) Target-DNA zu detektieren bzw. um Mutationen im Target aufzuspüren und sequenzspezifisch nachzuweisen. Dazu werden auf einer leitfähigen Oberfläche die Oberflächenatome oder -moleküle eines definierten Bereichs (einer Test-Site) mit DNA-/RNA-/PNA-Nukleinsäure-Oligomeren bekannter, aber beliebiger Sequenz, wie oben beschrieben, verknüpft. In einer allgemeinsten Ausführungsform kann aber der DNA-Chip auch mit einem einzigen Sonden-Oligonukleotid derivatisiert werden. Als Sonden- Nukleinsäure-Oligomere werden Nukleinsäure-Oligomere (z. B. DNA-, RNA- oder PNA-Fragmente) der Basenlänge 3 bis 50, bevorzugt der Länge 5 bis 30, besonders bevorzugt der Länge 8 bis 25 verwendet. Erfindungsgemäß wird oder ist an die Sonden-Nukleinsäure-Oligomere, wie nachfolgend beschrieben, eine redoxaktive Einheit gebunden.

Die Modifikation der Sonden-Nukleinsäure-Oligomere mit einer redoxaktiven Einheit kann komplett oder in Bestandteilen der redoxaktiven Einheit entweder vor oder nach der Bindung des Sonden-Oligonukleotids an die leitfähige Oberfläche erfolgen. Die verschiedenen Kombinationsmöglichkeiten der einzelnen Schritte (Reaktionssequenzen), sind mit Hilfe der Figur 2 am Beispiel einer über ein Sonden-Oligonukleotid an eine Elektrode gebundenen redoxaktiven Einheit im Abschnitt "Wege zur Ausführung der Erfindung" demonstriert.

Unabhängig von der jeweiligen Reaktionsseguenz entsteht ein Oberflächen-Hybrid der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit, wobei "Einheit" repräsentativ für die photoinduzierbar redoxaktive Einheit bzw. chemisch induzierbar redoxaktive Einheit steht. Die Verbrückungen können natürlich auch ohne Spacer oder mit nur einem Spacer (Elek-ss-oligo-Spacer-Einheit bzw. Elek-Spacer-ss-oligo-Einheit) durchgeführt werden. Im Beispiel der Figur 2 ist die Einheit eine photoinduzierbar redoxaktive Einheit, nämlich das Reaktionszentrum (RC) der Photosynthese betreibenden Bakterien des Stammes Rhodobacter sphaeroides, ein photoinduzierbar redoxaktives Protein bestehend aus Apoprotein und Cofaktoren. Im Beispiel der Figur 2, 3 und 4 ist das RC über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten QA-Protein-Bindungstasche des RCs kovalent mit dem Nukleinsäure-Oligomer verbunden. Das RC bildet mit dem Cofaktor Ubichinon-50 in der Q_A-Bindungstasche einen 1:1 Komplex, wobei das Ubichinon-50 in der beschriebenen Weise kovalent an das Nukleinsäure-Oligomer gebunden ist. Im Beispiel der Figur 5 und 6 ist die Einheit ein photoinduzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elektron-Donor-/Elektron-Akzeptor-Komplex, nämlich ein kovalen verknüpfter Zn-Bakteriochlorophyll-Chinon-Komplex, der über das Chinon, dem Eieltron-Akzeptor-Molekül des Komplexes, kovalent (über einen Spacer) mit dem Nukleinsäure-Oligomer verbunden ist.

Die elektrochemische Kommunikation zwischen der (leitfähigen) Oberfläche und der über ein Einzelstrang-Oligonukleotid verbrückten redoxaktiven Einheit ("Einheit") in der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit ist schwach oder gar nicht vorhanden.

In einem nächsten Schritt werden die Test-Sites mit der zu untersuchenden Nukleinsäure-Oligomer-Lösung (Target) in Kontakt gebracht. Dabei kommt es nur in dem Fall zur Hybridisierung, in dem die Lösung Nukleinsäure-Oligomer-Stränge enthält, die zu den an die leitfähige Oberfläche gebundenen Sonden-Nukleinsäure-Oligomeren komplementär, oder zumindest in weiten Bereichen komplementär sind. Im Falle der Hybridisierung zwischen Sonden- und Target-Nukleinsäure-Oligomer kommt es zu einer verstärkten Leitfähigkeit zwischen der Oberfläche und der redoxaktiven Einheit, da diese nunmehr über das aus einem Doppelstrang bestehende Nukleinsäure-Oligomer verbrückt ist. Figur 3 zeigt dies schematisch am Beispiel der Elek-Spacer-ss-oligo-Spacer-UQ(RC). In Figur 4 ist die Sequenz der Elektron-Transfer-Schritte in Elek-Spacer-ds-oligo-Spacer-UQ(RC) im Detail gezeigt, während Figur 5 das Beispiel Elek-Spacer-ss-oligo-Spacer-Q-ZnBChl schematisch zeigt und Figur 6 die Sequenz der Elektron-Transfer-Schritte in Elek-Spacer-ds-oligo-Spacer-Q-ZnBChl im Detail darstellt.

Aufgrund der Hybridisierung von Sonden-Nukleinsäure-Oligomer und dem dazu komplementären Nukleinsäure-Oligomer-Strang (Target) verändert sich die elektrische Kommunikation zwischen der (leitfähigen) Oberfläche und photoinduzierbar redoxaktiven Einheit. Somit kann ein sequenzspezifisches Hybridisierungsereignis durch elektrochemische Verfahren wie B. Cyclovoltametrie, Amperometrie oder Leitfähigkeitsmessungen detektiert werden.

Bei der Cyclovoltametrie wird das Potential einer stationären Arbeitselektrode zeitabhängig linear verändert. Ausgehend von einem Potential bei dem keine Elektrooxidation oder reduktion stattfindet, wird das Potential solange verändert bis die redoxaktive Substanz oxidiert oder reduziert wird (also Strom fließt). Nach Durchlaufen des Oxidations- bzw. Reduktionsvorgangs, der in der Strom/Spannungskurve einen zunächst ansteigenden Strom, dann einen Maximalstrom (Peak) und schließlich einen allmählich abfallenden Strom erzeugt, wird die Richtung des Potentialvorschubs umgekehrt. Im Rücklauf wird dann das Verhalten der Produkte der Elektrooxidation oder -reduktion aufgezeichnet.

Eine alternative elektrische Detektionsmethode, die Amperometrie, wird dadurch ermöglicht, daß die redoxaktive Einheit durch Anlegen eines geeigneten, konstant gehaltenen Elektrodenpotentials zwar elektrooxidiert (elektroreduziert) werden kann, die Rereduktion (Reoxidation) der redoxaktiven Einheit in den ursprünglichen Zustand aber nicht wie in der Cyclovoltametrie durch Änderung des Elektrodenpotentials erfolgt, sondern durch ein der Targetlösung zugesetztes geeignetes Reduktionsmittel (Oxidationsmittel), der "redoxaktiven Substanz", wodurch der Stromkreis des Gesamtsystems geschlossen wird. Solange solches Reduktionsmittel (Oxidationsmittel) vorhanden ist bzw. solange das verbrauchte Reduktionsmittel (Oxidationsmittel) an der Gegenelektrode rereduziert (reoxidiert) wird, fließt Strom, der amperometrisch detektiert werden kann und der proportional zur Zahl der Hybridisierungsereignisse ist.

Dieses Prinzip der amperometrischen Detektion soll stellvertretend für eine photoinduzierbar redoxaktive Einheit bzw. für eine redoxaktive Einheit am Beispiel der Glucoseoxidase näher erläutert werden. Die Glucoseoxidase ist ein aus Apoprotein und <u>einem</u> Cofaktor (Flavin-Adenin-Dinukleotid) bestehendes redoxaktives Enzym. Das mit einem Ende kovalent an die Elektrode angebundene Sonden-Oligonukleotid kann am anderen, noch freien Ende mit der vollständigen enzymatischen Einheit der Glucoseoxidase funktionalisiert werden, indem z. B. der Flavin-Adenin-Dinukleotid (FAD)-Cofaktor des Enzyms kovalent an das Sonden-Oligonukleotid angebunden wird und anschließend mit dem Glucoseoxidase-Apoprotein (GOx) rekonstituiert wird. Das entstandene Oberflächen-Hybrid der

Struktur Elek-Spacer-ss-oligo-Spacer-FAD(GOx) weist zwischen aligemeinen Elektrode und FAD keine oder nur geringe Leitfähigkeit auf. Im Falle der Hybridisierung mit dem zu "ss-oligo" komplementären Target-Oligonukleotid wird die Leitfähigkeit deutlich erhöht wird. Bei Zusatz des Substrats Glucose zur Target-Oligonukleotid-Lösung wird das FAD der Gukoseoxidase (FAD(GOx)) zu FADH2 der Glucoseoxidase (FADH2(GOx)) reduziert, wobei Glucose zur Gluconsäure oxidiert wird. Liegt nun an der Elektrode ein geeignetes äußeres Potential an, so daß über das hybridisierte Oligonukleotid Elektronen von FADH2(GOx) an die Elektrode abgegeben werden und somit FADH2(GOx) zu FAD(GOx) reoxidiert wird (aber weder Glucose noch Gluconsäure bei diesem Potential elektrooxidiert oder -reduziert werden kann), fließt im System Elek-Spacer-ds-oligo-Spacer-FAD(GOx) solange Strom wie FAD(GOx) durch freie Glucose reduziert wird, d. h. bis die gesamte Glucose verbraucht ist bzw. für den Fall, daß an der Gegenelektrode ein Potential anliegt, bei dem Gluconsäure zu Glucose reduziert werden kann, solange wie Gluconsäure an der Gegenelektrode reduziert wird. Dieser Strom kann detektiert werden amperometrisch und ist proportional Zahl der Hybridisierungsereignisse.

Die für diese Erfindung relevanten photoinduzierbar redoxaktiven Einheiten bzw. chemisch induzierbar redoxaktiven Einheiten besitzen statt eines Elektron-Donors bzw. Elektron-Akzeptors jedoch mindestens einen Elektron-Donor und mindestens einen Elektron-Akzeptor.

Im Falle einer chemisch induzierbar redoxaktiven Einheit ist im Sinne der vorliegenden Erfindung mindestens ein Ladungstransferschritt zwischen Elektron-Donor(en) und Elektron-Akzeptor(en) zwischengeschaltet. Die freie redoxaktive Substanz, die D reduziert (bzw. A oxidiert) und somit einen Elektrontransfer von Dzu A unter Bildung von A (bzw. den Elektrontransfer von Dzu A unter Bildung von Dinitiiert, ermöglicht es, die Elektrode auf ein Potential zu setzten, bei dem A (bzw. Dinitiiert, daß die Elektrode ein Potential besitzt, bei dem die direkte Reaktion der Vorteil, daß die Elektrode ein Potential besitzt, bei dem die direkte Reaktion der freien, redoxaktiven Substanz mit der Elektrode deutlich unterdrückt werden kann und hauptsächlich Elektronenübertragungen zwischen redoxaktiver Einheit und Elektrode detektiert werden.

Handelt es sich bei der redoxaktiven Einheit um eine photoinduzierbar redoxaktive Einheit, so wird die Redoxaktivität der Einheit erst durch Licht bestimmter oder beliebiger Wellenlänge ausgelöst. Erfindungsgemäß wird diese Eigenschaft dadurch ausgenutzt, daß die elektrochemische Detektion erst durch Einstrahlen von Licht auf

das Oberflächenhybrid der allgemeinen Struktur Elek-Spacer-ds-oligo-Spacer-Einheit (Oberflächenhybrid mit hybridisiertem Target) ausgelöst wird und maximal solange aufrechterhalten wird wie die Lichteinstrahlung andauert. Insbesondere bei der amperometrischen Detektion fließt somit bei Verwendung einer photoinduzierbar Einheit, unter bestimmten äußeren Umständen, redoxaktiven (längeranhaltend) Strom, wenn Licht auf das Oberfächenhybrid eingestrahlt wird. Solche äußere Umstände sind z. B. die Gegenwart eines geeigneten Reduktionsmittels (bzw. Oxidationsmittels), um einen durch Photoinduktion gebildeten, oxidierten Donor D* (bzw. reduzierten Akzeptor A-) der photoinduzierbar redoxaktiven Einheit zu reduzieren (bzw. zu reduzieren) und das Anliegen eines Potentials an der Elektrode, bei dem zwar ein durch Photoinduktion gebildeter reduzierter Akzeptor A⁻ (bzw. oxidierter Donor D⁺) der photoinduzierbar redoxaktiven Einheit, nicht jedoch der nicht reduzierte Akzeptor A (bzw. der nicht oxidierte Donor D) oxidiert (bzw. reduziert) werden kann. Im Abschnitt "Wege zur Ausführung der Erfindung" wird dies anhand verschiedener Beispiele von Elek-Spacer-ss-oligo-Spacer-Einheit mit photoinduzierbar redoxaktiver Einheit näher erläutert. Somit kann die Detektion bei Verwendung einer photoinduzierbar redoxaktiven Einheit auf eine bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe des Oligomer-Chips räumlich beschränkt werden, indem das Licht auf dieses Test-Site oder auf diese Test-Site-Gruppe begrenzt wird. Erfindungsgemäß können also verschiedene Test-Sites (Nukleinsäure-Oligomer-Kombinationen) eines Oligomer-Chips auf eine gemeinsame, durchgängige, elektrisch leitende Oberfläche aufgebracht werden. Ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe kann einfach durch Anlegen eines geeigneten äußeren Potentials an die (gesamte) Oberfläche bei Lichteinstrahlung auf genau dieses Test-Site oder diese Test-Site Gruppe adressiert und amperometrisch detektiert werden. Die verschiedenen Test-Sites müssen also nicht auf einzelnen, elektrisch voneinander isolierten und zum Anlegen eines Potentials und Auslesen des Stroms einzeln ansteuerbaren (Mikro-)Elektroden aufgebracht werden. Darüberhinaus kann bei der Verwendung Oberflächenhybriden der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit mit photoinduzierbar redoxaktiver Einheit und amperometrischer Detektion der Ausleseprozess zur Detektion der einzelnen sequenzspezifischen Hybridisierungsereignisse auf dem Oligomer-Chip dadurch optimiert werden, daß die Test-Sites durch entsprechende Fokusierung des Lichts erst grobgerastert ausgelesen werden und dann in den Rastern mit Hybridisierungsereignissen des Auflösungsvermögen sukzessive erhöht wird, also z. B. bei einem Oktamer-Chip mit 65.536 Test-Sites zuerst in z. B. 64 Gruppen von je 1024 Test-Sites ausgelesen wird, dann die Test-Site-Gruppen, die anhand der amperometrischen Messungen Hybridisierungsereignisse aufweisen, z. B. in 32 Gruppen von je 32 Test-Sites

durchgetestet werden und anschließend in den erneut Hybridisierungsereignisse aufweisenden Test-Site-Gruppen die Test-Sites einzeln ausgetestet werden. Die einzelnen Hybridisierungsereignisse können dadurch mit geringem experimentellen Aufwand schnell bestimmten Sonden-Oligomeren zugeordnet werden.

Kurze Beschreibung der Zeichnungen

Die Erfindung soll nachfolgend anhand von Ausführungsbeispielen im Zusammenhang mit den Zeichnungen näher erläutert werden. Es zeigen

- Fig. 1 Schematische Darstellung der Oligonukleotid-Sequenzierung durch Hybridisierung auf einem Chip;
- Fig. 2 Verschiedene Reaktionssequenzen zur Herstellung des Oberflächenhybrids Elek-Spacer-ss-oligo-Spacer-UQ(RC). Die photoinduzierbar redoxaktive Einheit in diesem Oberflächenhybrid ist das Reaktionszentrum (RC) der Photosynthese betreibenden Bakterien Rhodobacter sphaeroides. Dieses photoinduzierbar redoxaktive Protein besteht aus Apoprotein und Cofaktoren. Das RC ist über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten Q₄-Protein-Bindungstasche kovalent über einen Spacer mit dem Oligonukleotid verbunden:
- Schematische Darstellung der photoinduzierten amperometrischen Meßmethode am Beispiel des Oberflächen-Hybrids Elek-Spacer-ssoligo-Spacer-UQ(RC) aus Figur 2 (hv: Einstrahlung von Licht, P: primärer Donor des RC, UQ: Ubichinon-50 Elektron Akzeptor in der Q_A-Protein-Bindungstasche des RC, Red/Ox: reduzierte bzw. oxidierte Form der freien, der Targetlösung zugesetzten redoxaktiven Substanz, z. B. cyt c₂²⁺, Natriumascorbat oder Fe(ON)₆²⁺, die die oxidierte Form P⁺ in den ursprünglich neutralen Zustand P rereduzieren können, E_{Ox}: Potential der Elektrode, bei dem UQ⁻ durch Elektronabgabe an die Elektode zu UQ oxidiert wird, "hv an": Beginn der Lichteinstrahlung, "hv aus": Ende der Lichteinstrahlung);

Fig. 4 Detaillierte schematische Darstellung des Oberflächenhybrids Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) der Figur 3 mit Gold Oberflächenmaterial, Mercaptoethanol als Spacer (-S-CH₂CH₂- Spacer) zwischen Elektrode und Oligonukleotid und -CH2-CH=CH-CO-NH-CH2-CH₂-NH- als Spacer zwischen dem Elektron-Akzeptor UQ und Oligonukleotid sowie die Darstellung der Sequenz der photoinduzierten Elektron-Transfer-Schritte. Das Apoprotein des RCs ist nur als Hülle (durchgezogene Linie) angedeutet (vgl. Struktur 1). Das 12 Bp Sonden-Oligonukleotid der exemplarischen Sequenz 5'-TAGTCGGAAGCA-3' ist, als Ausschnitt, im hybridisierten Zustand gezeigt;

- Schematische Darstellung der photoinduzierten amperometrischen Meßmethode am Beispiel des Oberflächen-Hybrids Elek-Spacer-ssoligo-Spacer-Q-ZnBChl (hv: Einstrahlung von Licht, ZnBChl: das Elektron-Donor-Molekül Zn-Bakteriochlorophyll, Q: das Elektron-Akzeptor-Molekül Chinon, z. B. modifiziertes Anthrachinon oder PQQ, Red/Ox: reduzierte bzw. oxidierte Form der freien, der Targerkösung zugesetzten redoxaktiven Substanz, z. B. Fe(CN)₆²⁺, das die oxidierte Form des Elektron-Donors ZnBChl⁺ in den ursprünglich neutralen Zustand ZnBChl rereduzieren kann, E_{Ox}: Potential der Elektrode, bei dem Q⁻ durch Elektronabgabe an die Elektrode zu Q oxidiert wird, "hv an": Beginn der Lichteinstrahlung, "hv aus": Ende der Lichteinstrahlung);
- Fig. 6 Detaillierte schematische Darstellung des Oberflächenhybrids Au-S(CH₂)₂-ds-oligo-Spacer-Q-ZnBChl der Figur 5 mit Gold als Oberflächenmaterial, Mercaptoethanol als Spacer (-S-CH₂CH₂- Spacer) zwischen Elektrode und Oligonukleotid und -CH₂-CH=CH-CO-NH-CH₂-CH₂-NH- als Spacer zwischen dem Elektron-Akzeptor PQQ und Oligonukleotid sowie die Darstellung der Sequenz der photoinduzierten Elektron-Transfer-Schritte. Das 12 Bp Sonden-Oligonukleotid der exemplarischen Sequenz 5'-TAGTCGGAAGCA-3' ist, als Ausschnitt, im hybridisierten Zustand gezeigt.

Wege zur Ausführung der Erfindung

Eine Bildungseinheit einer exemplarischen Test-Site mit hybridisiertem Target, Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) der allgemeinen Struktur Elek-Spacer-ds-oligo-

Spacer-Einheit ist in Figur 4 dargestellt. Unter Bildungseinheit wird im Rahmen der vorliegenden Erfindung die kleinste sich wiederholende Einheit einer Test-Site verstanden. In dem Beispiel der Figur 4 ist die Oberfläche eine Gold-Elektrode. Die Verbindung zwischen Gold-Elektrode und Sonden-Oligonukleotid wurde mit dem Linker (HO-(CH₂)₂-S)₂ aufgebaut, der mit der endständigen Phosphatgruppe am 3' Ende zu P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert wurde und nach homolytischer Spaltung der S-S Bindung an der Gold-Oberfläche je eine Au-S Bindung bewirkte, womit 2-Hydroxymercaptoethanol und Mercaptoethanol-verbrücktes Oligonukleotid auf der Oberfläche koadsorbiert wurde. Die photoinduzierbar redoxaktive Einheit im Beispiel der Figur 4 ist das Reaktionszentrum (RC) der Photosynthese betreibenden Bakterien Rhodobacter sphaeroides, ein photoinduzierbar redoxaktives Protein bestehend aus Apoprotein und Cofaktoren. Im Anwendungsbeispiel ist das RC über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten QA-Bindungstasche des RCs kovalent mit dem Oligonukleotid verbunden, wobei zuerst freies UQ mit einer reaktiven Carbonsäuregruppe versehen wurde (siehe Beispiel 1), dann freies UQ über diese Carbonsäure-Gruppe kovalent an das Sonden-Oligonukleotid angebunden wurde (Amidbildung unter Wasserabspaltung mit der terminalen Aminofunktion des an die C-5-Position des 5'-Thymins angebundenen -CH=CH-CO-NH-CH₂-CH₂-NH₂ Linkers) und schließlich das restliche RC (Apoprotein mit allen Cofaktoren außer UQ) an UQ rekonstituiert wurde.

Wie weiter oben bereits erwähnt, kann die Modifikation der Sonden-Oligonukleotide mit der kompletten oder mit einem Bestandteil der redoxaktiven Einheit entweder vor oder nach der Bindung des Sonden-Oligonukleotids an die leitfähige Oberfläche erfolgen. Die verschiedenen Kombinationsmöglichkeiten der einzelnen Schritte, die prinzipiell zur selben Bildungseinheit einer Test-Site führen, sollen im folgenden mit Hilfe der Figur 2 am Beispiel des Oberflächenhybrids Au-S(CH₂)₂-ss-oligo-Spacer-UQ(RC) bzw. in seiner allgemeineren Form als Elek-Spacer-ss-oligo-Spacer-UQ(RC) dargestellt werden.

Das Reaktionszentrum kann durch einfache Manipulation von den beiden Ubichinon-Cofaktoren in der Q_A - bzw. Q_B -Bindungstasche befreit werden (Gunner, M.R., Robertson, D.E., Dutton, P.L.,1986, Journal of Physical Chemistry, Vol. 90, S. 3783-3795), so daß man Ubichinon getrennt vom restlichen RC (Apoprotein einschließlich aller Cofaktoren außer Ubichinon in der Q_A - bzw. Q_B -Bindungstasche) erhält. Das Sonden-Oligonukleotid ist in der Nähe der beiden Enden jeweils über einen (beliebigen) Spacer mit (gleichen oder verschiedenen) reaktiven Gruppe versehen. In einer Reaktionssequenz "1" kann das so modifizierte Sonden-Oligonukleotid in Gegenwart eines monofunktionalen Linkers (entsprechend den Punkten a) - c) und

2.) im Abschnitt "Bindung eines Oligonukleotids an die leitfähige Oberfläche") gemeinsam mit dem monofunktionalen Linker kovalent an die Elektrode angebunden werden, wobei darauf geachtet wird, daß genügend monofunktionaler Linker geeigneter Kettenlänge zugesetzt wird, um zwischen den einzelnen Sonden-Oligonukleotiden genügend Freiraum für eine Hybridisierung mit dem Target-Oligonukleotid und für die Anbindung der redoxaktiven Einheit zur Verfügung zu stellen. Danach wird an die freie, spacerverbrückte, reaktive Gruppe des Sonden-Oligonukleotids UQ, das vorher mit einer passenden reaktiven Kopplungsgruppe versehen wurde, angebunden. Die Anbindung erfolgt wie unter a) bzw. b) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben. Im letzten Schritt dieser Reaktionssequenz "1" wird dann das restliche RC (Apoprotein mit allen Cofaktoren außer UQ) an UQ rekonstituiert. In einer Variante dazu (Reaktionssequenz "2") kann das (mit Spacer und reaktiven Gruppen) modifizierte Sonden-Oligonukleotid zuerst ohne freien, monofunktionalen Linker (Spacer) kovalent an die Elektrode gebunden werden, wobei es zu einer flachen Anlagerung des Oligonukleotids kommt. Danach wird der freie, monofunktionale Linker (Spacer) kovalent an die Elektrode gebunden. Eine weitere Möglichkeit (Reaktionssequenz "3") besteht darin, das (mit Spacer und reaktiven Gruppen) modifizierte Sonden-Oligonukleotid zuerst mit UQ zu modifizieren, dann in Gegenwart von freiem, monofunktionalen Linker (Spacer) kovalent an die Elektrode anzubinden und anschließend mit dem restlichen RC zu rekonstituieren. Schließlich kann in einer Reaktionssequenz "4" das (mit Spacer und reaktiven Gruppen) modifizierte Sonden-Oligonukleotid zuerst mit UQ modifiziert werden, um es dann mit dem restlichen RC zu rekonstituieren und anschließend kovalent an die Elektrode zu binden. Falls, wie im Fall des RCs, die redoxaktive Einheit einen wesentlich größeren Durchmesser aufweist als das hybridisierte ds-Oligonukleotid (größer als 30 Å), kann auf die kovalente Anbindung eines geeigneten freien, monofunktionalen Linkers (Spacers) an die Elektrode verzichtet werden, anderenfalls geschieht die Anbindung der Struktur -Spacer-ss-oligo-Spacer-UQ(RC) an die Elektrode in Gegenwart eines geeigneten, freien monofunktionalen Linkers.

Im Beispiel der Figur 2 ist das RC über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten Q_A -Protein-Bindungstasche des RCs kovalent mit dem Oligonukleotid verbunden. Alternativ kann statt des UQ-Cofaktors in der Q_A -Bindungstasche auch ein anderer Cofaktor des RCs oder das Apoprotein kovalent an das Sonden-Oligonukleotid angebunden werden, es können beliebige Kombinationen der Reaktionssequenzen "1", "2", "3" oder "4" in Figur 2 angewandt werden, solange sie zum gleichen Endprodukt führen (vgl. Figur 2) und es kann in beliebigen Reaktionsschritten statt des Einzelstrang-Sonden-Oligonukleotids das mit

komplementären, unmodifizierten (Target-)Oligonukleotid hybridisierte Sonden-Oligonukleotid verwendet werden. Das Sonden-Oligonukleotid kann auch direkt, also nicht über einen Spacer verbrückt, sowohl an die Elektrode als auch an die redoxaktive Einheit angebunden werden, wie unter c) im Abschnitt "Bindung eines Nukleinsäure-Oligomers an die leitfähige Oberfläche" bzw. a) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben.

Die elektrische Kommunikation zwischen der leitfähigen Oberfläche und der über ein Einzelstrang-Oligonukleotid verbrückten redoxaktiven Einheit in der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit ist schwach oder gar nicht vorhanden. Durch Behandlung der Test-Site(s) mit einer zu untersuchenden Oligonukleotid-Lösung, kommt es, im Falle der Hybridisierung zwischen Sonde und Target, zu einer verstärkten Leitfähigkeit zwischen der Oberfläche und der über ein Doppelstrang-Oligonukleotid verbrückten redoxaktiven Einheit. Für die Bildungseinheit der exemplarische Test-Site Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) (mit 12-Bp Sonden-Oligonukleotiden) ist dies schematisch in Figur 3 anhand amperometrischer Messungen gezeigt.

Durch Lichteinstrahlung geeigneter Wellenlänge auf das RC wird der Cofaktor P, der sogenannte primäre Donor, elektronisch angeregt und es kommt innerhalb der Cofaktoren des RCs zur photoinduzierten Ladungstrennung, wobei ein Elektron vom angeregten primären Donor P* auf das UQ in der Q₄-Bindungstasche übertragen wird. Liegt an der Elektrode ein geeignetes Potential an, um vom reduzierten Ubichinon (UQ⁻) ein Elektron auf die Elektrode zu übertragen, kommt es im Falle des nicht mit Target-Oligonukleotid hybridisierten Sonden-Oligonukleotids trotzdem zu keinem Stromfluß, da die Leitfähigkeit des ss-Oligonukleotids in Au-S(CH₂)₂-ss-oligo-Spacer-UQ(RC) sehr gering oder überhaupt nicht vorhanden ist. Im hybridisierten Zustand (Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC)) jedoch ist die Leitfähigkeit hoch, ein Elektron kann von UQ- zur Elektrode übertragen werden (unter Bildung von UQ) und bei Anwesenheit einer geeigneten redoxaktiven Substanz, die P⁺ zu P zu reduziert, wird der Stromkreis geschlossen und weitere Lichtabsorption durch das RC startet den Zyklus erneut. Dies äußert sich amperometrisch in einem deutlichen Stromfluß zwischen Elektrode und photoinduzierbar redoxaktiver Einheit (Figur 3). Damit ist es möglich, die sequenzspezifische Hybridisierung des Targets mit den Sonden-Oligonukleotiden durch Amperometrie lichtinduziert zu detektieren. Die einzelnen Elektron Transfer Schritte, die im Oberflächenhybrid Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) durch Lichteinstrahlung und bei Anwesenheit einer geeigneten redoxaktiven Substanz zur Reduktion von P⁺ zu P ausgelöst werden, sind in Figur 4 dargestellt. Natürlich kann das Oberflächenhybrid Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) unter

geeigneten äußeren Umständen und bei geeigneter Anbindung (z. B. Anbindung des RCs an das Sonden-Oligonukleotid in der Nähe des primären Donors), auch umgekehrt geschaltet werden, so daß nach Lichteinstrahlung P* von der Elektrode reduziert und Q- von einem geeigneten Oxidationsmittel oxidiert wird.

Eine weiteres Test-Site, Au-S(CH₂)₂-ss-oligo-Spacer-Q-ZnBChI, der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit ist in Figur 5 dargestellt. Durch Lichteinstrahlung geeigneter Wellenlänge auf ZnBChl wird ZnBChl elektronisch angeregt und es kommt zur photoinduzierten Ladungstrennung, wobei ein Elektron vom angeregten ZnBChl* auf das Chinon Q übertragen wird. Liegt an der Elektrode ein geeignetes Potential an, um vom so reduzierten Chinon (Q-) ein Elektron auf die Elektrode zu übertragen, kommt es im Falle des nicht mit Target-Oligonukleotid hybridisierten Sonden-Oligonukleotids trotzdem zu keinem Stromfluß, da die Leitfähigkeit des ss-Oligonukleotids in Au-S(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl sehr gering oder überhaupt nicht vorhanden ist. Im hybridisierten Zustand Au-S(CH₂)₂-dsoligo-Spacer-Q-ZnBChl jedoch ist die Leitfähigkeit hoch, ein Elektron kann von Qzur Elektrode übertragen werden (unter Bildung von Q) und bei Anwesenheit einer geeigneten redoxaktiven Substanz, die ZnBChl⁺ zu ZnBChl reduziert, wird der Stromkreis geschlossen und weitere Lichtabsorption durch ZnBChl startet den Zyklus erneut. Dies äußert sich amperometrisch in einem deutlichen Stromfluß zwischen Elektrode und photoinduzierbar redoxaktiver Einheit (Figur 5). Damit ist es möglich, die sequenzspezifische Hybridisierung des Targets mit den Sonden-Oligonukleotiden durch Amperometrie lichtinduziert zu detektieren. Natürlich Oberflächenhybrid Au-S(CH₂)₂-ds-oligo-Spacer-Q-ZnBChl unter geeigneten äußeren Umständen und geeigneter Anbindung (z. B. Au-S(CH₂)₂-ds-oligo-Spacer-ZnBChl-Q) auch umgekehrt geschaltet werden, so daß nach Lichteinstrahlung ZnBChl* von der Elektrode reduziert und Q von einem geeigneten Oxidationsmittel oxidiert wird.

Da die Redoxaktivität der photoinduzierbar redoxaktiven Einheit - auch bei passendem Elektrodenpotential - erst durch Lichteinstrahlung geeigneter Wellenlänge ausgelöst und maximal solange aufrechterhalten wird, wie die Lichteinstrahlung andauert, kann dies erfindungsgemäß dadurch ausgenutzt werden, daß ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe eines Oligomer-Chips räumlich aufgelöst wird, indem das Licht auf dieses Test-Site oder auf diese Test-Site-Gruppe begrenzt wird. Dies birgt den erfindungsgemäßen Vorteil, daß die verschiedenen Test-Sites (Nukleinsäure-Oligomer-Kombinationen) eines Oligomer-Chips auf eine gemeinsame, durchgängige, elektrisch leitende Oberfläche aufgebracht werden können und ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppen einfach durch Anlegen eines geeigneten äußeren Potentials an die

(gesamte) Oberfläche bei Lichteinstrahlung nur auf genau dieses Test-Site oder diese Test-Site Gruppe adressiert und amperometrisch detektiert werden kann. Die verschiedenen Test-Sites müssen also nicht auf einzelnen, elektrisch voneinander isolierten und zum Anlegen eines Potentials und Auslesen des Stroms einzeln ansteuerbaren (Mikro-) Elektroden aufgebracht werden.

Daneben können fehlerhafte Basenpaarungen (Basenpaar Mismatches) durch eine geänderte cyclovoltammetrische Charakteristik erkannt werden. Ein Mismatch äußert sich in einem größeren Potentialabstand zwischen den Strommaxima der Elektroreduktion und der Elektroreoxidation (Umkehrung der Elektroreduktion bei umgekehrter Potentialvorschubrichtung) bzw. der Elektrooxidation und Elektrorereduktion in einem cyclovoltammetrisch reversiblen Elektronen-Transfer zwischen der elektrisch leitenden Oberfläche und der photoinduzierbar redoxaktiven Einheit. Dieser Umstand wirkt sich vor allem in der amperometrischen Detektion günstig aus, da dort der Strom bei einem Potential getestet werden kann, bei dem zwar das perfekt hybridisierende Oligonukleotid-Target signifikant Strom liefert, nicht aber das fehlerhaft gepaarte Oligonukleotid-Target.

Beispiel 1: Modifikation des Ubichinon-50 mit einer Spacer-verbrückten reaktiven Carbonsäure-Gruppe. Die 2-Methoxy-Gruppe des Ubichinon-50 (UQ-50) wird durch Etherspaltung mit HBr, einer Standardmethode, zur 2-Hydroxygruppe modifiziert (alternativ kann 2-OH-UQ-50 nach dem Verfahren von Moore, H. W. and Folkers, K. Journal of the American Chemical Society, 1966, 88, 564-570 oder von Daves, G. et al., Journal of the American Chemical Society, 1968, 90, 5587-5593 hergestellt werden). Anschließend wird 2-OH-UQ-50 in einem Standardverfahren mit einer äquimolaren Menge an CI-CH2-CH2-CO2H zum 2-(CH2-CH2-CO2H)-UQ-50 umgesetzt chromatographisch aufgereinigt. Alternativ können 5-OH-6-alkyl-1,4-Benzochinon-Analoga des UQ-50 (Darstellung gemäß Catiin et al., Journal of the American Chemical Society, 1968, 90, 3572-3574) in einem Standardverfahren mit einer äquimolaren Menge an CI-CH₂-CH₂-CO₂H zu 5-(CH₂-CH₂-CO₂H)-UQ-50-Analoga modifiziert werden.

Beispiel 2: Herstellung der Oligonukleotid-Elektrode Au-S(CH₂)₂-ss-oligo-SpacerUQ(RC). Die Herstellung von Au-S(CH₂)₂-ss-oligo-Spacer-UQ(RC) gliedert sich in 4 Teilabschnitte, nämlich der Darstellung der leitfähigen Oberfläche, der Derivatisierung der Oberfläche mit dem Sonden-Oligonukleotid in Gegenwart eines geeigneten monofunktionalen Linkers (Inkubationsschritt), der kovalenten Anbindung des modifizierten Ubichinon-50 (Redoxschritt) und der Rekonstitution des restlichen RCs (Rekonstitutionsschritt).

Das Trägermaterial für die kovalente Anbindung der Doppelstrang-Oligonukleotide bildet ein ca. 100 nm dünner Gold-Film auf Mica (Muskovit Plättchen). Dazu wurde in einer elektrischen Entladungskammer frisch gespaltenes Mica mit einem Argon-Ionenplasma gereinigt und durch elektrische Entladung Gold (99.99%) in einer Schichtdicke von ca. 100nm aufgebracht. Anschließend wurde der Gold-Film mit 30 % $<math>\mbox{H}_2\mbox{O}_2$, / 70 % $\mbox{H}_2\mbox{SO}_4$ von Oberflächenverunreinigungen befreit (Oxidation organischer Ablagerungen) und für ca. 20 Minuten in Ethanol getaucht, um an der Oberfläche adsorbierten Sauerstoff zu verdrängen. Nach Abspülen der Oberfläche mit bidestilliertem Wasser wird auf die horizontal gelagerte Oberfläche eine vorher bereitete 1×10^{-4} molare Lösung des (modifizierten) Doppelstrang-Oligonukleotids aufgetragen, so daß die komplette Gold-Oberfläche benetzt wird (Inkubationsschritt, siehe auch unten).

Zur Inkubation wurde ein doppelt modifiziertes 12 Bp Einzelstrang-Oligonukleotid der Sequenz 5'-TAGTCGGAAGCA-3' verwendet, das an der Phosphatgruppe des 3' Endes mit (HO-(CH₂)₂-S)₂ zum P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert ist. Am 5'-Ende ist die endständige Base Thymin des Oligonukleotids am C-5 Kohlenstoff mit -CH=CH-CO-NH-CH₂-CH₂-NH₂ modifiziert. Zu einer 2x10⁻⁴ molaren Lösung dieses Oligonukleotids in HEPES-Puffer (0,1 molar in Wasser, pH 7.5 mit 0.7 molarem Zusatz von TEATFB, siehe Abkürzungen) wurde ca. 10⁻⁴ bis 10⁻¹ molar 2-Hydroxy-mercaptoethanol gegeben (oder ein anderer Thiol- oder Disulfid-Linker geeigneter Kettenlänge) und die Gold-Oberfläche eines Test-Sites komplett benetzt und 2-24h inkubiert. Während dieser Reaktionszeit wird der Disulfidspacer P-O-(CH₂)₂-S-S-(CH₂)₂-OH des Oligonukleotids homolytisch gespalten. Dabei bildet der Spacer mit Au-Atomen der Oberfläche eine kovalente Au-S Bindung aus, wodurch es zu einer 1:1 Koadsorption des ss-Oligonukleotids und des abgespaltenen 2-Hydroxy-mercaptoethanols kommt. Das in der Inkubationslösung gleichzeitig anwesende, freie 2-Hydroxy-mercaptoethanol wird ebenfalls durch Ausbildung einer Au-S Bindung koadsorbiert (Inkubationsschritt).

Die so mit einer Monolayer aus ss-Oligonukleotid und 2-Hydroxy-mercaptoethanol modifizierte Goldelektrode wurde mit bidestilliertem Wasser gewaschen und anschließend mit einer Lösung von 3x10⁻³ molarem Chinon 2-(CH₂-CH₂-CO₂H)-UQ-50, 10⁻² molarem EDC und 10⁻² molarem sulfo-NHS in HEPES-Puffer (0,1 molar (in Wasser, pH = 7.5), benetzt. Nach einer Reaktionszeit von ca. 1 - 4 h bilden der - CH=CH-CO-NH-CH₂-CH₂-NH₂ Spacer und das 2-(CH₂-CH₂-CO₂H)-UQ-50 eine kovalente Bindung aus (Amidbildung zwischen der Aminogruppe des Spacers und der C-2-Säurefunktion des 2-(CH₂-CH₂-CO₂H)-UQ-50, Redoxschritt).

Anschließend wurde die so modifizierte Goldelektrode mit bidestilliertem Wasser gewaschen und mit einer Lösung von ca. 5x10⁻⁵ molarem Ubichinon-50-freien RCs in 10 mM Tris, pH = 8, mit 0.7 molarem Zusatz von TEATFB bei ca. 4 °C für ca. 12 h inkubiert, um das restliche RC an das Oligonukleotid-gebundene UQ-50 zu rekonstituieren (Rekonstitutionsschritt).

Alternativ zur kovalenten Anbindung von 2-(CH₂-CO₂H)-UQ-50 an das Sonden-Oligonukleotid kann, unter gleichen Bedingungen, auch ein 5-(CH₂-CO₂H)-UQ-50-Analogon (Beispiel 1) oder ein anderes, mit einer reaktiven Carbonsäure versehenes Chinon der Formel 1 - 8 verwendet werden, da auch an diese Ubichinon-50-freies RC rekonstituiert werden kann.

Beispiel 3: Herstellung der Oligonukleotid-Elektrode Au-S(CH2)2-ss-oligo-Spacer-Q-ZnBChl. Die Herstellung von Au-S(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl gliedert sich in 5 Teilabschnitte. nämlich der Darstellung der leitfähigen Oberfläche, Derivatisierung der Oberfläche mit dem (mit Komplementärstrang hybridisierten) Sonden-Oligonukleotid in Gegenwart eines geeigneten monofunktionalen Linkers (Inkubationsschritt). der kovalenten **Anbindung** des[,] Elektron-Akzeptors (Akzeptorschritt), der kovalenten Anbindung des Elektron-Donors (Donorschritt) und der thermischen Dehybridisierung des Doppelstrang-Oligonukleotids (Dehybridisierungsschritt).

Das Trägermaterial für die kovalente Anbindung der Doppelstrang-Oligonukleotide, ein ca. 100 nm dünner Gold-Film auf Mica (Muskovit Plättchen), wurde wie in Beispiel 1 beschrieben, hergestellt.

Zur Inkubation wurde ein doppelt modifiziertes 12 Bp Einzelstrang-Oligonukleotid der Sequenz 5'-TAGTCGGAAGCA-3' verwendet, das an der Phosphatgruppe des 3' Endes mit $(HO-(CH_2)_2-S)_2$ zum P-O- $(CH_2)_2-S-S-(CH_2)_2-OH$ verestert ist. Am 5'-Ende ist die endständige Base Thymin des Oligonukleotids am C-5 Kohlenstoff mit -CH=CH-CO-NH- CH_2 - CH_2 - NH_2 modifiziert. Eine $2x10^{-4}$ molare Lösung dieses Oligonukleotids im Hybridisierungspuffer (10 mM Tris, 1 mM EDTA, pH 7.5 mit 0.7 molarem Zusatz von TEATFB, siehe Abkürzungen) wurde mit einer 2x10⁻⁴ molaren Lösung des (unmodifizierten) komplementären Strangs im Hybridisierungspuffer bei Raumtemperatur für ca. 2h hybridisiert (Hybridisierungsschritt). Nach Hybridisierung wurde der nun 1x10⁻⁴ molaren Doppelstrang-Oligonukleotid-Lösung ca. 10⁻⁴ bis 10⁻¹ molar 2-Hydroxy-mercaptoethanol (oder ein anderer Thiol- oder Disulfid-Linkers geeigneter Kettenlänge) zugesetzt, die Gold-Oberfläche eines Test-Sites komplett benetzt und 2-24h inkubiert. Während dieser Reaktionszeit wird der Disulfidspacer P-O-(CH₂)₂-S-S-(CH₂)₂-OH des Oligonukleotids homolytisch gespalten. Dabei bildet der Spacer mit Au-Atomen der Oberfläche eine kovalente Au-S Bindung aus, wodurch es zu einer 1:1 Koadsorption des ds-Oligonukleotids und des abgespaltenen 2-Hydroxy-mercaptoethanols kommt. Das in der Inkubationslösung gleichzeitig anwesende, freie 2-Hydroxy-mercaptoethanol wird ebenfalls durch Ausbildung einer Au-S Bindung koadsorbiert (Inkubationsschritt).

Die so mit einer Monolayer aus ds-Oligonukleotid und 2-Hydroxy-mercaptoethanol modifizierte Goldelektrode wurde mit bidestilliertem Wasser gewaschen und anschließend mit einer Lösung von 3x10⁻³ molarem Chinon PQQ, 10⁻² molarem EDC und 10⁻² molarem sulfo-NHS in HEPES Puffer benetzt. Nach einer Reaktionszeit von ca. 1 - 4 h bilden der -CH=CH-CO-NH-CH₂-CH₂-NH₂ Spacer und das PQQ eine

kovalente Bindung (Amidbildung zwischen der Aminogruppe des Spacers und der C-7-Carbonsäurefunktion des PQQ, Akzeptorschritt).

Anschließend wurde die so modifizierte Goldelektrode mit bidestilliertem Wasser gewaschen und mit einer wässerigen Lösung aus 3x10⁻³ molarem Donor ZnBChl (freie Säure), 1,5x10⁻¹ molarem EDC, 2,5x10⁻³ molarem Hydrazin-Monohydrat (NH2-NH2xH2O) und 1x10-1 molarem Imidazol benetzt. Nach einer Reaktionszeit von ca. 16 h bei 23 °C bindet die C-1-Carbonsäurefunktion des an das Oligonukleotid gebundenen PQQ über Hydrazin an die freie Carbonsäure-Gruppe des ZnBChl (Amidbildung zwischen den Aminogruppen des Hydrazins und der C-1-Carbonsäure-Gruppe des PQQ bzw. der freien Carbonsäure-Gruppe des ZnBChl, Donorschritt). Anschließend wurden die Doppelstränge bei Temperaturen von T > 40 °C thermisch dehybridisiert und erneut mit bidestilliertem Wasser abgespült (Dehybridisierungsschritt). Das ZnBChl (freie Säure) wird aus Zn-BChl (Darstellung gemäß Hartwich et al., Journal of the American Chemical Society, 1998, 120, 3684-3693) durch Inkubation mit Trifluoressigsäure hergestellt.

(Probe-Oligonukleotid-gebundenes) Q

Alternativ kann z. B. ZnBChl (freie Säure) über Esterbildung nach Standardverfahren auch an die 3-OH-Gruppe des 5'-terminalen Zuckers des Sonden-Oligonukleotids gebunden werden oder der vorher kovalent verbundene Elektron-Donor/Elektron-Akzeptor-Komplex wird, wie im Donorschritt beschrieben, über eine freie Carbonsäure-Gruppe z. B. des Donors an das Sonden-Oligonukleotid angebunden. Statt PQQ kann unter den gleichen Reaktionsbedingungen auch Anthrachinon-2,6-Disulfonsäure Dinatriumsalz im Akzeptorschritt verwendet werden. Bei Verwendung PNA-Oligonukleotid mit z. B. -NH-(CH₂)₂-N(COCH₂-Base)-CH₂CO- als von Oligonukleotid-Baustein besteht eine alternative Anbindungsmöglichkeit der ZnBChl-PQQ-Einheit an das Nukleinsäure-Oligomer (PNA-Oligonukleotid) entsprechend d) im Abschnitt "Bindung einer photoinduzierbar redoxaktiven Einheit an ein Nukleinsäure-Oligomer". Dabei wird während der PNA-Oligonukleotid-Synthese statt der N-terminale Base in der Standard-PNA-Synthese-Reaktion PQQ über seinen Pyrrol-Stickstoff angebunden. Anschließend wird Zn-BChl, ähnlich wie im Donorschritt beschrieben durch Inkubation des mit PQQ modifizierten PNA-

Oligonukleotids mit 3x10⁻³ molarem ZnBChI (freie Säure), 1,5x10⁻¹ molarem EDC 10⁻² und 2x10⁻¹ molarem sulfo-NHS in HEPES Puffer an das Amino-Ende des Peptid-Rückgrats gebunden (Amidbildung zwischen der Aminogruppe des Rückgrats und der Carbonsäure-Gruppe des Zn-BChI (freie Säure)).

Patentansprüche

- Durch kovalente Anbindung einer redoxaktiven Einheit modifiziertes Nukleinsäure-Oligomer, dadurch gekennzeichnet, daß die redoxaktive Einheit ein oder mehrere Elektron-Donor-Moleküle und ein oder mehrere Elektron-Akzeptor-Moleküle enthält.
- 2.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 1, dadurch gekennzeichnet, daß die redoxaktive Einheit wenigstens einen redoxaktiven, verknüpften, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplex enthält, wobei wenigstens zwei der Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-Molekül(e) der redoxaktiven Einheit durch eine oder mehrere Bindungen miteinander verbunden sind.
- 3.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 2, dadurch gekennzeichnet, daß wenigstens zwei der Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-Molekül(e) der redoxaktiven Einheit durch eine oder mehrere kovalente Bindungen miteinander verbunden sind.
- 4.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 1, dadurch gekennzeichnet, daß die redoxaktive Einheit wenigstens einen redoxaktiven, verknüpften, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplex enthält, wobei wenigstens zwei der Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-Molekül(e) durch einen oder mehrere verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent verbunden sind.
- 5.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 4, wobei die verzweigten oder unverzweigten Molekülteile eine Kettenlänge von 1 20 Atomen, insbesondere 1 14 Atomen, aufweisen.
- 6.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die ein oder mehrere Elektron-Donor-Moleküle und ein oder mehrere Elektron-Akzeptor-Moleküle enthaltende redoxaktive Einheit zusätzlich ein oder mehrere Makromoleküle umfaßt.
- 7.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei die redoxaktive Einheit das native oder modifizierte

Reaktionszentrum von Photosynthese betreibenden Organismen ist, insbesondere das native oder modifizierte Reaktionszentrum von Photosynthese betreibenden Bakterien.

- 8.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß eines oder mehrere der Elektron-Donor-und/oder Elektron-Akzeptor-Molekül(e) Farbstoffe sind, insbesondere Flavine, (Metallo-)Porphyrine, (Metallo-)Chlorophylle oder (Metallo-)Bakteriochlorophylle bzw. Derivate davon.
- 9.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß eines oder mehrere der Elektron-Donor-und/oder Elektron-Akzeptor-Molekül(e) Nikotinsäureamide oder Chinone sind, insbesondere Pyrrolo-Chinolin-Chinone (PQQ), 1,2-Benzochinone, 1,4-Benzochinone, 1,2-Naphtochinone, 1,4-Naphtochinone oder 9,10-Anthrachinone bzw. Derivate davon.
- 10.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß eines oder mehrere der Elektron-Donor-und/oder Elektron-Akzeptor-Molekül(e) Charge-Transfer-Komplexe sind.
- 11.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 10, wobei der Charge-Transfer-Komplex ein Übergangsmetall-Komplex ist, insbesondere ein Ru(II)-, ein Cr(III)-, ein Fe(II)-, ein Os(II)- oder ein Co(II)-Komplex.
- 12.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei das modifizierte Nukleinsäure-Oligomer sequenzspezifisch Einzelstrang-DNA, RNA und/oder PNA binden kann.
- 13.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 12, wobei das modifizierte Nukleinsäure-Oligomer ein Desoxyribonukleinsäure-, Ribonukleinsäure-, ein Peptidnukleinsäure-Oligomer oder ein Nukleinsäure-Oligomer mit strukturell analogem Rückgrat ist.
- 14.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei die redoxaktive Einheit kovalent alternativ an eine der Phosphorsäure-, Carbonsäure- oder Amin-Gruppen oder an einen Zucker, insbesondere an eine Zucker-Hydroxy-Gruppe, des Nukleinsäure-Oligomer-Rückgrats gebunden ist.

15.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 - 13, wobei die redoxaktive Einheit alternativ kovalent an eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.

- 16.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 15, dadurch gekennzeichnet, daß die reaktive Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe der Base kovalent über einen verzweigten oder unverzweigten Molekülteil beliebiger Zusammensetzung und Kettenlänge an die Base gebunden ist, wobei die kürzeste durchgehende Verbindung zwischen der Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe und der Base ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen, insbesondere von 1 14 Atomen, ist.
- 17.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 14 16, wobei die redoxaktive Einheit an ein Ende des Nukleinsäure-Oligomer-Rückgrats bzw. an eine endständige, modifizierte Base angebunden ist.
- 18.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die redoxaktive Einheit eine photoinduzierbar redoxaktive Einheit ist.
- 19.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 17, dadurch gekennzeichnet, daß die redoxaktive Einheit eine chemisch induzierbar redoxaktive Einheit ist.
- 20.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß mehrere redoxaktive Einheiten an das Nukleinsäure-Oligomer angebunden sind.
- 21.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers wie in einem der vorhergehenden Ansprüche definiert, wobei eine redoxaktive Einheit kovalent an ein Nukleinsäure-Oligomer angebunden wird.
- 22.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 21, wobei die redoxaktive Einheit durch kovalente Anbindung eines oder mehrerer Elektron-Donor-Molekül(e) an ein Nukleinsäure-Oligomer angebunden wird.

23.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 21, wobei die redoxaktive Einheit durch kovalente Anbindung eines oder mehrerer Elektron-Akzeptor-Molekül(e) an ein Nukleinsäure-Oligomer angebunden wird.

- 24.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 21, wobei die redoxaktive Einheit durch kovalente Anbindung eines oder mehrerer Makromoleküle bzw. durch kovalente Anbindung eines oder mehrerer Proteine an ein Nukleinsäure-Oligomer angebunden wird.
- 25.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach den Ansprüchen 22 24, wobei die redoxaktive Einheit durch Zugabe von einem oder mehreren Elektron-Akzeptor-Molekül(en), einem oder mehreren Elektron-Donor-Molekül(en), einem oder mehreren Makromolekülen und/oder einem oder mehreren Proteinen vervollständigt wird.
- 26.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 21 25, wobei das Nukleinsäure-Oligomer alternativ durch eine oder mehrere Amidbildungen mit Amin- oder mit Säure-Gruppen der redoxaktiven Einheit, durch eine oder mehrere Esterbildungen mit Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit, durch Thioesterbildung mit Thio-Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit bzw. durch Kondensation einer oder mehrerer Amin-Gruppen des Nukleinsäure-Oligomers mit Aldehyd-Gruppen der redoxaktiven Einheit und anschließender Reduktion der entstandenen Kohlenstoff-Stickstoff-Doppelbindung an die redoxaktive Einheit gebunden wird.
- 27.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 21 26, wobei an die redoxaktive Einheit kovalent eine oder mehrere verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge angebunden ist und die verzweigten oder unverzweigten Molekülteile alternativ eine reaktive Amin-, Hydroxy-, Thiol-, Säure- oder Aldehyd-Gruppe zur kovalenten Anbindung an ein Nukleinsäure-Oligomer besitzen.
- 28.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 27, wobei die kürzeste durchgehende Verbindung zwischen dem Nukleinsäure-Oligomer und der redoxaktiven Einheit ein verzweigtes oder

unverzweigtes Molekülteil mit einer Kettenlänge von 1 - 20 Atomen, insbesondere von 1 - 14 Atomen, ist.

- 29.) Modifizierte leitfähige Oberfläche, dadurch gekennzeichnet, daß eine oder mehrere Arten von modifizierten Nukleinsäure-Oligomeren gemäß einem der Ansprüche 1 bis 20 an eine leitfähige Oberfläche angebunden sind.
- 30.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einem Metall oder einer Metallegierung besteht, insbesondere einem Metall ausgewählt aus der Gruppe Platin, Palladium, Gold, Cadmium, Quecksilber, Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium, Mangan und deren Mischungen.
- 31.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einem Halbleiter besteht, insbesondere einem Halbleiter ausgewählt aus der Gruppe Kohlenstoff, Silizium, Germanium und -Zinn.
- 32.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einer binären Verbindung der Elemente der Gruppen 14 und 16, einer binären Verbindung der Elemente der Gruppen 13 und 15, einer binären Verbindung der Elemente der Gruppen 15 und 16, oder einer binären Verbindung der Elemente der Gruppen 11 und 17 besteht, insbesondere aus einem Cu(I)-Halogenid oder einem Ag(I)-Halogenid.
- 33.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einer ternären Verbindung der Elemente der Gruppen 11, 13 und 16 oder einer ternären Verbindung Elemente der Gruppen 12, 13 und 16 besteht.
- 34.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 29 33, wobei die Anbindung der modifizierten Nukleinsäure-Oligomere an die leitfähige Oberfläche kovalent oder durch Chemi- bzw. Physisorption erfolgt.
- 35.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 34, wobei alternativ eine der Phosphorsäure-, Carbonsäure-, Amin- oder eine Zucker-Gruppe, insbesondere eine Zucker-Hydroxy-Gruppe, des Nukleinsäure-Oligomer-Rückgrats kovalent oder durch Chemi- bzw. Physisorption an die leitfähige Oberfläche angebunden ist.

36.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 - 34, dadurch gekennzeichnet, daß alternativ eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers kovalent oder durch Chemi- bzw. Physisorption an die leitfähige Oberfläche angebunden ist.

- 37.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 35 oder 36, wobei das modifizierte Nukleinsäure-Oligomer über eine Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats bzw. über eine Gruppe einer endständigen, modifizierten Base an die leitfähige Oberfläche gebunden ist.
- 38.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 29 37, wobei an die leitfähige Oberfläche verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent oder durch Chemi- bzw. Physisorption angebunden sind und die modifizierten Nukleinsäure-Oligomere kovalent an diese Molekülteile angebunden sind.
- 39.) Modifizierte leitfähige Oberfläche nach Anspruch 38, wobei die kürzeste durchgehende Verbindung zwischen der leitfähigen Oberfläche und dem Nukleinsäure-Oligomer ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen, insbesondere von 1 12 Atomen, ist.
- 40.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 38 oder 39, wobei der verzweigte oder unverzweigte Molekülteil alternativ an eine Phosphorsäure-, Carbonsäure-, eine Amin- oder eine Zucker-Gruppe, insbesondere eine Zucker-Hydroxy-Gruppe, des Nukleinsäure-Oligomer-Rückgrats oder eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.
- 41.) Modifizierte leitfähige Oberfläche nach Anspruch 40, wobei der verzweigte oder unverzweigte Molekülteil an eine Phosphorsäure-, Zucker-Hydroxy-, Carbonsäure- oder Amin-Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats bzw. eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer endständigen, modifizierten Base gebunden ist.
- 42.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 41, dadurch gekennzeichnet, daß jeweils überwiegend eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.

43.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 - 41, dadurch gekennzeichnet, daß jeweils ausschließlich eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.

- 44.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 29 43 definiert, dadurch gekennzeichnet, daß ein oder mehrere Arten von modifizierten Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht werden.
- 45.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 29 43 definiert, dadurch gekennzeichnet, daß ein oder mehrere Arten von Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht werden und anschließend eine Modifikation der Nukleinsäure-Oligomere durch ein Verfahren gemäß den Ansprüchen 21 28 durchgeführt wird.
- 46.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach Anspruch 44 oder 45, wobei die Nukleinsäure-Oligomere oder die modifizierten Nukleinsäure-Oligomere mit dem dazu jeweils komplementären Nukleinsäure-Oligomerstrang hybridisiert werden und in Form des Doppelstranghybrids auf die leitfähige Oberfläche aufgebracht werden.
- 47.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 44 oder 45, wobei das Nukleinsäure-Oligomer oder das modifizierte Nukleinsäure-Oligomer in Gegenwart von weiteren chemischen Verbindungen, die ebenfalls an die leitfähige Oberfläche angebunden werden, auf die leitfähige Oberfläche aufgebracht wird.
- 48.) Verfahren zur elektrochemischen Detektion von Oligomer-Hybridisierungsereignissen, dadurch gekennzeichnet, daß eine oder mehrere modifizierte leitfähige Oberflächen, wie in den Ansprüchen 29 - 43 definiert, mit Nukleinsäure-Oligomeren in Kontakt gebracht werden und anschließend eine Detektion der elektrischen Kommunikation zwischen der redoxaktiven Einheit und der jeweiligen leitfähigen Oberfläche erfolgt.
- 49.) Verfahren nach Anspruch 48, wobei die Detektion cyclovoltametrisch, amperometrisch oder durch Leitfähigkeitsmessung erfolgt.

50.) Verfahren zur elektrochemischen Detektion nach den Ansprüchen 48 oder 49, dadurch gekennzeichnet, daß die elektrochemische Detektion durch photoinduzierte Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktive Einheit gestartet wird.

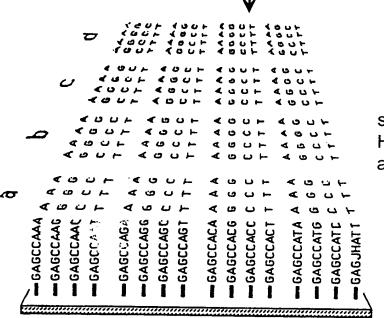
- 51.) Verfahren nach Anspruch 50, wobei die Lichteinstrahlung zur photoinduzierten Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktive Einheit auf einen Bereich der leitfähigen Oberfläche mit einer oder mehreren modifizierten Nukleinsäure-Oligomeren-Arten begrenzt wird.
- 52.) Verfahren nach einem der Ansprüche 50 oder 51, wobei das nach Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entstandene oxidierte Elektron-Donor-Molekül oder das entstandene reduzierte Elektron-Akzeptor-Molekül der photoinduzierbar redoxaktiven Einheit durch eine geeignete, freie, nicht an das Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz, re-reduziert bzw. re-oxidiert, also jeweils in seinen ursprünglichen vor der Lichteinstrahlung vorhandenen Zustand, zurückversetzt wird.
- 53.) Verfahren zur elektrochemischen Detektion nach den Ansprüchen 48 oder 49, dadurch gekennzeichnet, daß die elektrochemische Detektion durch eine freie redoxaktive Substanz, durch die ein chemisch induzierter Ladungstransfer auf die redoxaktive Einheit stattfindet, ermöglicht wird.
- 54.) Verfahren nach Anspruch 52 oder 53, wobei die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz bei einem Potential selektiv oxidierbar und reduzierbar ist, wobei der Bedingung 2,0 V 2,0 V, gemessen gegen Normalwasserstoffelektrode, genügt.
- 55.) Verfahren nach einem der Ansprüche 52 54, wobei die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz ein freies Chinon, ein freier Hexacyanoferrat(II)-Komplex, ein freies Natriumascorbat, ein freier Ru(II)hexamin-Komplex oder ein freies redoxaktives Protein, insbesondere ein freies Cytochrom, ist.



Fig. 1

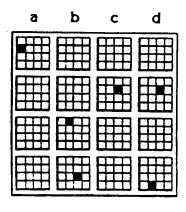
DNA-Fragment

5'-... AGTCCCTTGGCTC...-3'



spezifische Hybridisierung an Oktamermatrix

Sequanzbestimmung durch Mustererkennung



3'-TCAGGGAA-5'
3'-CAGGGAAC-5'
3'-AGGGAACC-5'
3'-GGGAACCG-5'
3'-GGAACCGA-5'
3'-GAACCGAG-5'

Oktamer 1
Oktamer 2
Oktamer 3
Oktamer 4
Oktamer 5
Oktamer 6



3'-TCAGGGAACCGAG-5'

zusammengesetzte Komplementärsequenz



5'-...AGTCCCTTGGCTC...-3' deduzierte Sequenz des DNA-Fragments

Fig. 2

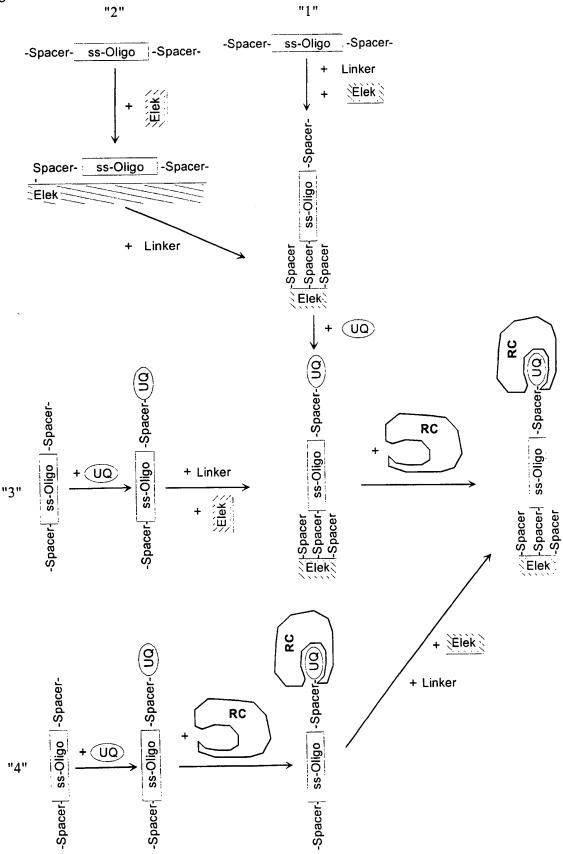
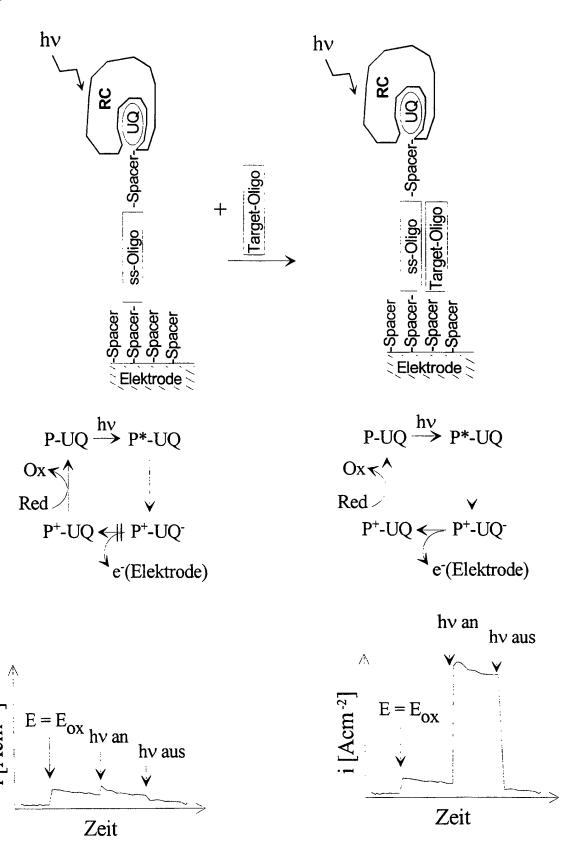
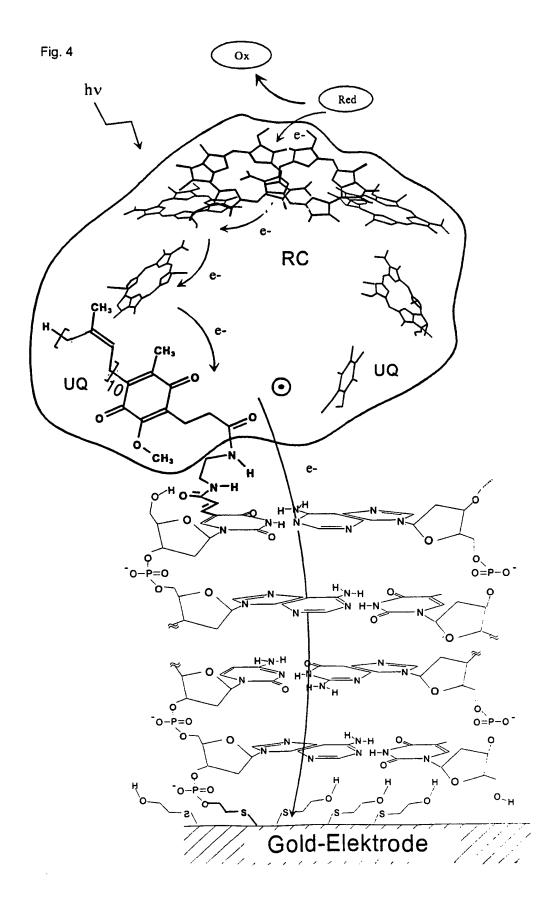
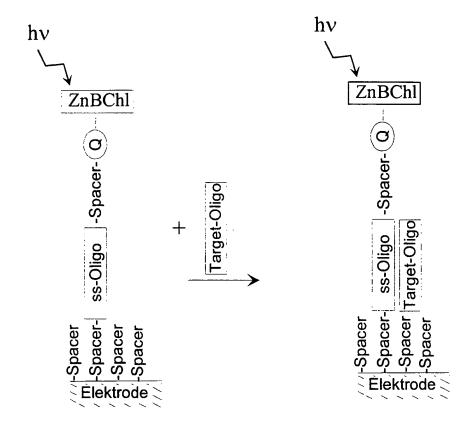
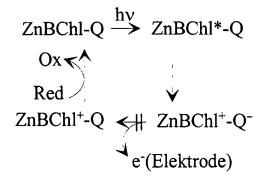


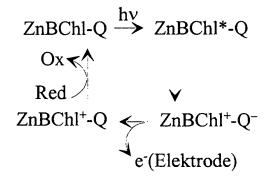
Fig. 3

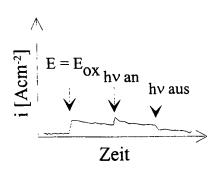












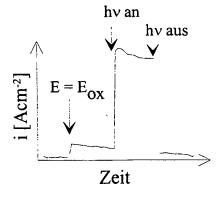
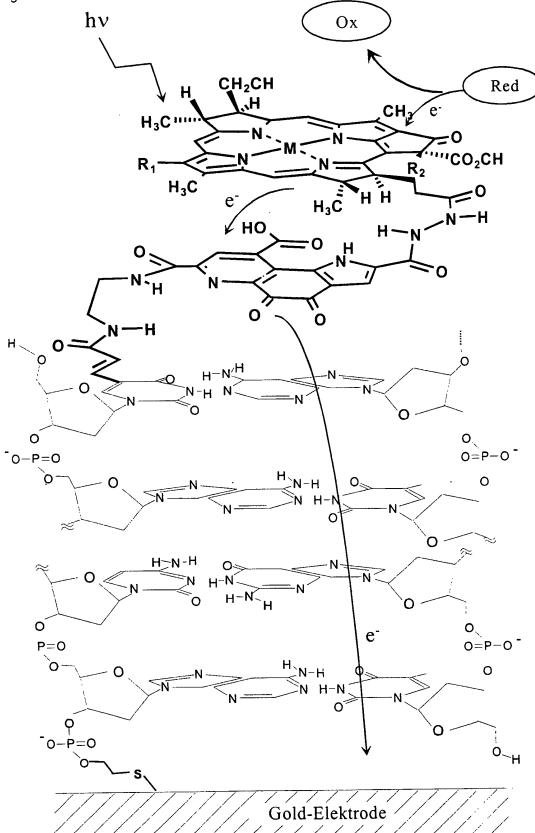


Fig. 5

Fig. 6



Translation

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference FRI006/00	FOR FURTHER ACTION		ionofTransmittalofInternational Preliminary Report (Form PCT/IPEA/416)	
International application No.	International filing date (da)	/month/year)	Priority date (day/month/year)	
PCT/EP00/00084	07 January 2000 (0	7.01.00)	18 January 1999 (18.01.99)	
International Patent Classification (IPC) or n C12Q 1/68, C07H 21/00	ational classification and IPC			
Applicant	HARTWICH, Ge	rhard		
 This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36. This REPORT consists of a total of 5 heets, including this cover sheet. 				
This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have be amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see R 70.16 and Section 607 of the Administrative Instructions under the PCT). These annexes consist of a total of 10 sheets.			on, claims and/or drawings which have been tions made before this Authority (see Rule	
These afflexes consist of a to	tal 01 Slicets.			
3. This report contains indications rela	ting to the following items:			
Basis of the report				
II Priority				
III Non-establishment o	of opinion with regard to nove	ty, inventive ste	p and industrial applicability	
IV Lack of unity of inv	ention			
v Reasoned statement citations and explan	under Article 35(2) with regar ations supporting such stateme	d to novelty, in nt	ventive step or industrial applicability;	
VI Certain documents of	cited			
VII Certain defects in th	e international application			
VIII Certain observations on the international application				
Date of submission of the demand	Date	of completion o	f this report	
09 July 2000 (09.07.	00)	21 N	March 2001 (21.03.2001)	
Name and mailing address of the IPEA/EP	Auth	orized officer		
Facsimile No.	Tele	hone No.		

International application No.

PCT/EP00/00084

1.	Basis	of the re	port	
1. With regard to the elements of the international application:*				
		the inter	mational application as originally filed	
	\boxtimes	the desc	ription:	
İ		pages	1-61	, as originally filed
		pages		, filed with the demand
		pages	, filed with the letter of	
	\square	the clair	ms:	
		pages		, as originally filed
		pages	, as amended (together with any stat	
		pages		, filed with the demand
		pages	1-71, filed with the letter of07 November	er 2000 (07.11.2000)
	\square	the drav	vings:	
		pages	1/6.6/6	, as originally filed
		pages	1/0-0/0	
		pages	, filed with the letter of	
	П.	- 		
	Ш,	•	nce listing part of the description:	:-:-:
		pages -		
		pages	, filed with the letter of	
		•		
2.	the ir	nternation	the language, all the elements marked above were available or furnished to this Authority in all application was filed, unless otherwise indicated under this item. It is were available or furnished to this Authority in the following language	the language in which which is:
			guage of a translation furnished for the purposes of international search (under Rule 23.1(b)).	
		the lang	guage of publication of the international application (under Rule 48.3(b)).	
		the lang	guage of the translation furnished for the purposes of international preliminary examination.	(under Rule 55.2 and/
3.			to any nucleotide and/or amino acid sequence disclosed in the international applica amination was carried out on the basis of the sequence listing:	tion, the international
		contain	ed in the international application in written form.	
		filed to	gether with the international application in computer readable form.	
	\boxtimes	furnishe	ed subsequently to this Authority in written form.	
	\boxtimes	furnishe	ed subsequently to this Authority in computer readable form.	
	\boxtimes		atement that the subsequently furnished written sequence listing does not go beyond ional application as filed has been furnished.	the disclosure in the
			tement that the information recorded in computer readable form is identical to the written rnished.	n sequence listing has
4.		The am	endments have resulted in the cancellation of:	
			the description, pages	
			the claims, Nos.	
			the drawings, sheets/fig	
5.			ort has been established as if (some of) the amendments had not been made, since they have the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**	been considered to go
*	in th	acement s is report 70.17).	theets which have been furnished to the receiving Office in response to an invitation under Ar. as "originally filed" and are not annexed to this report since they do not contain am	ticle 14 are referred to endments (Rule 70.16
**			ent sheet containing such amendments must be referred to under item 1 and annexed to this rep	ort.

nternational application No.

PCT/EP 00/00084

1.	Basis of the report
1.	This report has been drawn on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):
	The sequence listing submitted with the letter of 7 July
	2000 does not form part of the application (PCT Rule
	13ter.1(f)).
ļ	
l	

International application No.
PCT/EP 00/00084

. Reasoned statement under Article . citations and explanations supporti	35(2) with regard to novelty ng such statement	inventive step or industrial app	licability;
Statement			<u> </u>
Novelty (N)	Claims	1-71	YES
	Claims		NO
Inventive step (IS)	Claims	1-71	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-71	YES
	Claims		NO

Citations and explanations

Document US-A-5 770 369 (D1) discloses nucleic acid 1. oligomers modified by covalent binding to redoxactive compounds, such as transition metal complexes (abstract). The transition metal complexes may contain, inter alia, the metals Cd, Mg, Cu, Co, Pd, Zn, Fe, Ru, Rh, Os, Re, Pt, Sc, Ti, V, Cr, Mn, Ni, Mo, Tc, W and Ir (column 7, lines 35-48). As metal ligands, D1 mentions, inter alia, pyridine, pyrazine, bipyridine, phenanthroline, 9,10-phenanthrenequinone diimine, porphyrin and substituted porphyrin derivatives (column 7, lines 49-62). Alternatively to the transition metal complexes, other organic electron donors or acceptors may also be covalently bound to the nucleic acids (column 8, lines 25-27), for example riboflavin, quinones, porphyrins... (column 8, lines 27-43).

According to one embodiment of D1, the nucleic acids are bound to an electrode (column 8, lines 47-50), allowing electrons to be transported directly via a covalent bond between the electrode and the nucleic acid (column 9, lines 56-58).

D1 therefore provides a system for binding an oligonucleotide modified with a redox active organic molecule to an electrode (column 11, lines 1-12). The presence of double-stranded DNA can be determined by comparing the electron transfer rates of the non-hybridised sample and of the hybridised sample (column 12, lines 44-48). Amperometry is proposed, inter alia, for detecting electron transfer, which allows the current strengths f hybridised and non-hybridised DNA to be compared (column 27, lines 20-47). Irradiation with light can be used to initiate electron transfer (column 23, lines 50-53).

1.1 Document WO-A-98/20162 (D2) discloses nucleic acids modified by covalent binding to redox active compounds, such as transition metal complexes (page 37, line 22; and page 25, lines 27-33). Pyridine, pyrazine, bipyridine, phenanthroline..., inter alia, are indicated as metal ligands (page 26, lines 14-29). Alternatively to the transition metal complexes, other organic electron donors or acceptors may be covalently bound to the nucleic acids (page 37, lines 29-31), such as riboflavin, quinones, porphyrins... (page 37, line 30 - page 38, line 5).

The modified nucleic acids are bound to an electrode using a conductive polymer (Claim 1), so that electron transport can occur between the electrode and the nucleic acid (Claim 9).

D2 therefore provides a system for binding an oligonucleotide modified with a redox active organic molecule to an electrode using a conductive polymer

(Claims 1 and 9). The presence of double-stranded DNA can be detected by the increased electron transfer from DNA to electrode (page 52, lines 21-24). Amperometry is proposed, inter alia, for detecting electron transfer (page 56, lines 18-25).

- 1.2 Document DE-A-42 16 696 (D3) discloses a method for carrying out assays involving, inter alia, interactions between DNA strands. Detection is based, for example, on current strength measurement, using stable redox-labelled analyte molecules (abstract). For cnat purpose, a DNA strand is covalently bound to a stable redox system (column 4, lines 6-14). Ferrocene derivatives, ruthenium complexes, hydroquinones, hexacyanoferrate (II/III), iodine/iodide or the like are proposed as redox systems (e.g. Claim 22).
- 1.3 None of the documents D1-D3 and none of the available prior art documents discloses or suggests a nucleic acid oligomer modified by a redox active unit in which the redox active unit, as required by independent Claim 1 of the present application, contains at least one electron donor molecule and at least one electron acceptor molecule, and in which the electron donor molecule and the electron acceptor molecule are not interconnected by a nucleic acid oligomer (see also page 10, lines 18-19, of the description).
- 1.4 The advantages of such a modified nucleic acid oligomer over the modified nucleic acid oligomers described in the prior art are, *inter alia*, that a charge is transmitted in the excited state directly to another molecule within the redox active unit,

and not directly to the DNA. Only then is the charge transmitted by the acceptor to the DNA. The advantage of these successive transmissions resides in the persistence of the state of the redox active unit after the charge is first transmitted. This is at least 10³ times longer, in the case of the photosynthetic bacterial reaction centre (page 32), or even 10⁹ times longer than that of the originally excited donor state. The probability of charge transmission to the DNA increases by the same factor, having drastic effects on the intensity of the current led through the DNA.

- 1.5 Claim 1 is therefore novel and inventive (PCT Article 33(2) and (3)).
- Dependent Claims 2-27 define further specific embodiments of Claim 1 and are therefore also novel and inventive (PCT Article 33(2) and (3)).
- 2. Claims 28-36 define methods for producing a modified nucleic acid oligomer as described in Claims 1-27. Since these modified nucleic acid oligomers are novel and inventive, the methods described in Claims 28-36 are also novel and inventive (PCT Article 33(2) and (3)).
- 3. The same applies to the modified conductive surfaces defined in Claims 37-57 and characterised in that at least one modified nucleic acid oligomer as per Claims 1-27 is bound to such a conductive surface.

Claims 37-57 therefore meet the requirements of PCT Article 33(2) and (3) for novelty and inventive step.

International application No.
PCT/EP 00/00084

4. In an analogous manner, the methods defined in Claims 58-71 for producing such modified conductive surfaces are also novel and inventive (PCT Article 33(2) and (3)).

International application No.

PCT/EP00/00084

cen	tain documents cited					
erta	in published document	s (Rule 70.10)				
	Application No. Patent No.	Publicatio (day/mont)		Filing date (day/month/year,	, _	Priority date (valid claim (day/month/year)
	WO-A-99 51778	14 October 199	9 (14.10.1999)	07 April 1999 (07.()4.1999)	08 April 1998 (08.04.19
	WO-A-00 31101	02 June 2000	(02.06.2000)	19 November 1999 (1	9.11.1999)	23 November 1998 (23.11.
						29 April 1999 (29.04.19
n-w	ritten disclosures (Rule	70.9)				
n-w	ritten disclosures (Rule Kind of non-written d		Date of non-ur	itten disalogues	Date of	f written disclosure
n-w	ritten disclosures (Rule Kind of non-written d		Date of non-wr	itten disclosure tth/year)	referring to	written disclosure non-written disclosure ny/month/year)
n-w			Date of non-wr	itten disclosure tth/year)	referring to	non-written disclosure
n-w			Date of non-wr	itten disclosure uth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure tth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure nth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure tth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure nth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure tth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure nth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure tth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure hth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure th/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure uth/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure th/year)	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure	referring to	non-written disclosure
n-w			Date of non-wr (day/mor	itten disclosure	referring to	non-written disclosure
n-w			Date of non-wr	itten disclosure	referring to	non-written disclosure

International application No. PCT/EP 00/00084

Sun	plemen	tal Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: BOX VI

When the application enters the regional phase, the above-mentioned documents could become relevant to the assessment of novelty.

The validity of the priority claim has not been examined.

Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 7:

C12O 1/68

(11) Internationale Veröffentlichungsnummer: A2

WO 00/42217

(43) Internationales

Veröffentlichungsdatum:

20. Juli 2000 (20.07.00)

(21) Internationales Aktenzeichen:

PCT/EP00/00084

(22) Internationales Anmeldedatum:

7. Januar 2000 (07.01.00)

(30) Prioritätsdaten:

199 01 761.1 199 26 457.0

18. Januar 1999 (18.01.99) 29. April 1999 (29.04.99)

DE DE

(71)(72) Anmelder und Erfinder: HARTWICH, Gerhard [DE/DE]: Nibelungenstrasse 10, D-80639 München (DE).

(81) Bestimmungsstaaten: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, IP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht

Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(54) Title: METHOD FOR ELECTROCHEMICALLY DETECTING NUCLEIC ACID-OLIGOMER HYBRIDISATION EVENTS

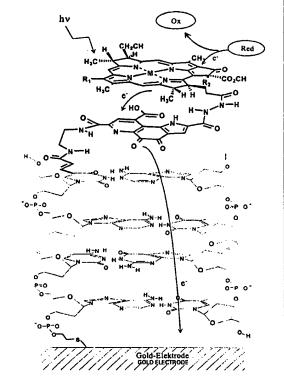
(54) Bczeichnung: VERFAHREN ZUR ELEKTROCHEMISCHEN DETEKTION VON NUKLEINSÄURE -OLIGOMER- HYBRI-DISIERUNGSEREIGNISSEN

(57) Abstract

The invention relates to a method for electrochemically detecting sequence-specific nucleic acid-oligomer hybridisation events. DNA/RNA/PNA oligomer single strands which are bound to a conductive surface at one end and linked to a redoxactive unit at the other, free end, serve as a hybridisation matrix (probe). A proportion of the single strand oligonucleotides are hybridised by treatment with the oligonucleotide solution (target) being tested, with the result that the electrical communication between the conductive surface and the redoxactive unit, which is initially non- or barely existent, is increased. This enables a hybridisation event to be detected using electrochemical methods such as voltammetry, amperometry or conductance measurement.

(57) Zusammenfassung

vorliegende Erfindung betrifft Verfahren ein zur elektrochemischen Detektion von sequenzspezifischen Nukleinsäure-Oligomer-Hybridisierungsereignissen. Dahei dienen DNA-/RNA-/PNA-Oligomer-Einzelstränge, die mit einem Ende an einer leitfähigen Oberfläche gebunden und am anderen, freien Ende mit einer redoxaktiven Einheit verknüpft sind, als Hybridisierungsmatrix (Sonde). Durch Behandlung mit der zu untersuchenden Oligonukleotid-Lösung (Target) wird ein Teil der Einzelstrang-Oligonukleotide hybridisiert, wodurch die ursprünglich nicht oder nur schwach vorhandene elektrische Kommunikation zwischen der leitfähigen Oberfläche und der redoxaktiven Einheit erhöht wird. Somit wird die Detektion eines Hybridisierungsereignisses durch elektrochemische Verfahren wie Voltametrie, Amperometrie oder Leitfähigkeitsmessung ermöglicht.



្រី ÷



PCT

INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts	WEITERES VORGEHEN				
Internationales Aktenzeichen	Internationales Anme (Tag/Monat/Jahr)	ldedatum	(Frühestes) Prioritätsdatum (Tag/Monat/Jahr)		
PCT/EP 00/00084	1 ' 7		18/01/1999		
Anmelder					
HARTWICH, Gerhard					
Dieser internationale Recherchenbericht wurd Artikel 18 übermittelt. Eine Kopie wird dem In	de von der International ternationalen Būro über	en Recherchenbehörde e rmittelt.	erstellt und wird dem Anmelder gemäß		
Dieser internationale Recherchenbericht umfa X Darüber hinaus liegt ihm jev	aßt insgesamt <u>3</u> veils eine Kopie der in d	Blätter. diesem Bericht genannten	n Unterlagen zum Stand der Technik bei.		
Grundlage des Berichts			mattendan kamatahan ta dan Oranga		
 a. Hinsichtlich der Sprache ist die inte durchgeführt worden, in der sie eing 	ernationale Recherche a gereicht wurde, so िता र	 æ der Grundlage der inte inter diesem Punkt nichts 	ernationalen Anmeidung in der Sprache anderes angegeben ist.		
Die internationale Recherch Anmeldung (Regel 23.1 b))	ne ist auf der Grundk ja durchgeführt worden.	: einer bei der Behörde ein	ngereichten Übersetzung der internationalen		
Recherche auf der Grundlage des S in der internationalen Anme zusammen mit der internati X bei der Behörde nachträglic X bei der Behörde nachträglic X Die Erklärung, daß das nac internationalen Anmeldung	Sequenzprotokolls durch eldung in Schriftlicher Form eich in schriftlicher Form eich in computerlesbarer lehträglich eingereichte sim Anmeldezeitpunkt homputerlesbarer Form eich eiben sich als nicht rect der Erfindung (siehe ndung gereichte Wortlaut gener	hgeführt worden, das orm enthalten ist. omputerlesbarer Form ein eingereicht worden ist. Form eingereicht worden i schriftliche Sequenzprotok inausgeht, wurde vorgeleg erfaßten Informationen der cherchlerbar erwiesen (si Feld II).	ist. koll nicht über den Offenbarungsgehalt der gt. m schriftlichen Sequenzprotokoll entsprechen,		
Anmelder kann der Behörd Recherchenberichts eine S	legel 38.2b) in der in Fe de innerhalb eines Mona Stellungnahme vorlegen	eld III angegebenen Fassu ats nach dem Datum der A n.	ung von der Behörde festgesetzt. Der Absendung dieses internationalen		
6. Folgende Abbildung der Zelchnungen Wie vom Anmelder vorgeso		assung zu veröffentlichen	: Abb. Nrkeine der Abb.		
weil der Anmelder selbst k	_	hlagen hat.			
weil diese Abbildung die E					

Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybridisierungsereignissen

Technisches Gebiet

Die vorliegende Erfindung betrifft ein modifiziertes Nukleinsäure-Oligomer, sowie ein Verfahren zur elektrochemischen Detektion von sequenzspezifischen Nukleinsäure-Oligomer-Hybridisierungsereignissen.

Stand der Technik

Zur Sequenzanalyse von DNA und RNA, z. B. in der Krankheitsdiagnose, bei toxikologischen Testverfahren, in der genetischen Forschung und Entwicklung, sowie auf dem Agrar- und pharmazeutischen Sektor, werden im allgemeinen gelelektrophoretische Verfahren mit autoradiographischer oder optischer Detektion verwendet.

Beim wichtigsten gel-elektrophoretischen Verfahren mit optischer Detektion, dem Sanger-Verfahren wird eine DNA enthaltende Lösung in vier Ansätze aufgeteilt. Zur Unterscheidung der vier Ansätze ist der Primer (komplementäre Startsequenz zur Replikation) jedes Ansatzes mit je einem bei verschiedener Wellenlänge emitierenden Fluoreszenzfarbstoff kovalent modifiziert. Ausgehend vom Primer wird jeder Ansatz durch DNA-Polymerase I enzymatisch repliziert. Neben den dazu nötigen Desoxyribonucleosid-Triphosphaten der Basen A (Adenin), T (Thymin), C (Cytosin), und G (Guanin) enthält jedes Reaktionsgemisch noch genügend 2',3'-Didesoxyanalogon eines dieser Nukleosidtriphosphate als Stopbase (je eine der 4 möglichen Stopbasen pro Ansatz), um die Replikation an allen möglichen Bindungsstellen zu stoppen. Nach Vereinigung der vier Ansätze entstehen replizierte DNA-Fragmente aller Längen mit stopbasenspezifischer Fluoreszenz, die gelelektrophoretisch der Länge nach sortiert und durch Fluoreszenz-Spektroskopie charakterisiert werden können.

Ein anderes optisches Detektionsverfähren basiert auf der Anlagerung von Fluoreszenzfarbstoffen wie z. B. Ethidiumbromid an Oligonukleotide. Im Vergleich zur freien Lösung des Farbstoffs ändert sich die Fluoreszenz solcher Farbstoffe bei Assoziation mit doppelsträngiger DNA oder RNA drastisch und kann deshalb zum Nachweis hybridisierter DNA oder RNA verwendet werden.

Bei der radioaktiven Markierung wird ³²P in das Phosphatgerüst der Oligonukleotide eingebaut, wobei ³²P gewöhnlich am 5'-Hydroxylende durch Polynukleotid-Kinase addiert wird. Die markierte DNA wird anschließend an jeweils einem der vier Nukleotidtypen bevorzugt gespalten und zwar unter definierten Bedingungen, so daß pro Kette durchschnittlich eine Spaltung erfolgt. Damit liegen im Reaktionsgemisch für einen bestimmten Basentyp Ketten vor, die sich von der ³²P-Markierung bis zur Position dieser Base erstrecken (bei mehrfachem Auftreten der Base erhält man entsprechend Ketten unterschiedlicher Länge). Die vier Fragmentgemische werden anschließend auf vier Bahnen gel-elektrophoretisch aufgetrennt. Danach wird vom Gel ein Autoradiogramm angefertigt, an dem die Sequenz unmittelbar abgelesen werden kann.

Vor einigen Jahren wurde ein weiteres, auf optischer (oder autoradiographischer) Detektion beruhendes Verfahren zur DNA-Sequenzierung entwickelt, nämlich die Sequenzierung durch Oligomer-Hybridisierung (vgl. z. B. Drmanac et al., Genomics 4, (1989), S. 114-128 oder Bains et al., Theor. Biol. 135, (1988), S. 303-307). Bei diesem Verfahren wird ein vollständiger Satz kurzer Oligonukleotide Nukleinsäure-Oligomere (Sonden-Oligonukleotide), z. B. alle 65536 möglichen Kombinationen der Basen A, T, C und G eines Oligonukleotid-Oktamers auf ein Trägermaterial gebunden. Die Anbindung geschieht in einem geordneten Raster aus 65536 Test-Sites, wobei jeweils eine größere Menge einer Oligonukleotid-Kombination ein Test-Site definieren und die Position jeder einzelnen Test-Site (Oligonukleotid-Kombination) bekannt ist. Auf solch einer Hybridisierungsmatrix, dem Oligomer-Chip, wird ein DNA-Fragment, dessen Sequenz man ermitteln will (das Target), mit Fluoreszenzfarbstoff (oder ³²P) markiert und unter Bedingungen, die nur eine spezifische Doppelstrangbildung erlauben, hybridisiert. Dadurch bindet das Target DNA-Fragment nur an die Nukleinsäure-Oligomere (im Beispiel an die Oktamere), deren komplementäre Sequenz exakt einem Teil (einem Oktamer) seiner eigenen Sequenz entspricht. Durch optische (oder autoradiographische) Detektion der Bindungsposition des hybridisierten DNA-Fragments werden damit alle im Nukleinsäure-Oligomersequenzen (Oktamersequenzen) Fragment vorhandenen Nukleinsäurebenachbarter Überlappung der Aufarund bestimmt. Oligomersequenzen kann durch geeignete mathematische Algorithmen die fortlaufende Sequenz des DNA-Fragments bestimmt werden. Die Vorteile dieses Verfahrens liegen unter anderem in der Miniaturisierung der Sequenzierung und damit in der enormen Datenmenge, die gleichzeitig in einem Arbeitsgang erfaßt wird. Daneben kann auf Primer und auf das gel-elektrophoretische Auftrennen der DNA-Fragmente verzichtet werden. Beispielhaft ist dieses Prinzip in Figur 1 für ein 13 Basen langes DNA-Fragment gezeigt.

Die Verwendung radioaktiver Markierungen bei der DNA-/RNA-Sequenzierung ist mit mehreren Nachteilen verbunden, wie z. B. aufwendige, gesetzlich vorgeschriebene Sicherheitsvorkehrungen beim Umgang mit radioaktiven Materialien, die Strahlenbelastung, das begrenzte räumliche Auflösungsvermögen (maximal 1mm²) und eine Sensitivität, die nur dann hoch ist, wenn die Strahlung der radioaktiven Fragmente entsprechend lange (Stunden bis Tage) auf einen Röntgenfilm einwirkt. Es kann zwar die räumliche Auflösung durch zusätzliche Hard- und Software erhöht und die Detektionszeit durch die Verwendung von β -Scannern verkürzt werden, beides ist jedoch mit erheblichen zusätzlichen Kosten verbunden.

Die Fluoreszenzfarbstoffe, die üblicherweise zur Markierung der DNA verwendet werden, sind zum Teil (z. B. Ethidiumbromid) mutagen und erfordern, ebenso wie die Anwendung der Autoradiographie, entsprechende Sicherheitsvorkehrungen. In fast allen Fällen erfordert die Verwendung optischer Detektion den Gebrauch von einem oder mehreren Lasersystemen und somit geschultes Personal und entsprechende Sicherheitsvorkehrungen. Die eigentliche Detektion der Fluoreszenz erfordert zusätzliche Hardware, wie z. B. optische Bauelemente zur Verstärkung und, bei verschiedenen Anregungs- und Abfragewellenlängen wie im Sanger-Verfahren, ein Kontrollsystem. Abhängig von den benötigten Anregungswellenlängen und der gewünschten Detektionsleistung können somit erhebliche Investitionskosten entstehen. Bei der Sequenzierung durch Hybridisierung auf dem Oligomer-Chip ist die Detektion noch (kosten)aufwendiger, da, neben dem Anregungssystem, zur 2-dimensionalen Detektion der Fluoreszenzspots hochauflösende CCD-Kameras (Charge Coupled Device Kameras) benötigt werden.

Obwohl es also quantitative und extrem sensitive Methoden zur DNA-/RNA-Sequenzierung gibt, sind diese Methoden zeitaufwendig, bedingen aufwendige Probenpräparation und teure Ausstattung und sind im allgemeinen nicht als transportable Systeme verfügbar.

Darstellung der Erfindung

Aufgabe der vorliegenden Erfindung ist es deshalb, eine Vorrichtung und ein Verfahren zur Detektion von Nukleinsäure-Oligomer-Hybriden zu schaffen, welche die Nachteile des Standes der Technik nicht aufweisen.

Diese Aufgabe wird erfindungsgemäß durch das modifizierte Nukleinsäure-Oligomer gemäß unabhängigem Patentanspruch 1, durch das Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers gemäß unabhängigem Anspruch 21, durch die modifizierte leitfähige Oberfläche gemäß unabhängigem Patentanspruch 29, das Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche gemäß unabhängigem Patentanspruch 43 und ein Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybridisierungsereignissen gemäß unabhängigen Patentanspruch 48 gelöst.

Im Rahmen der vorliegenden Erfindung werden die folgenden Abkürzungen und Begriffe benutzt:

Genetik	
DNA	Desoxyribonukleinsäure
RNA	Ribonukleinsäure
PNA	Peptidnukleinsäure (synthetische DNA oder RNA, bei der die Zucker-Phosphat Einheit durch eine Aminosäure ersetzt ist. Bei Ersatz der Zucker-Phosphat Einheit durch die -NH-(CH ₂) ₂ -N(COCH ₂ -Base)-CH ₂ CO- Einheit hybridisiert PNA mit DNA.)
Α	Adenin
G	Guanin
С	Cytosin
Т	Thymin
U	Uracil
Base	A, G, T, C oder U
Вр	Basenpaar
Nukleinsäure	wenigstens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- (z. B. Cytosin, Thymin oder Uracil) oder Purin-Basen (z. B. Adenin oder Guanin). Der Begriff Nukleinsäure bezieht sich auf ein beliebiges "Rückgrat" der kovalent verbundenen Pyrimidinoder Purin-Basen, wie z. B. auf das Zucker-Phosphat Rückgrat der DNA, cDNA oder RNA, auf ein Peptid-Rückgrat der PNA oder auf analoge Strukturen (z. B. Phosphoramid-,

4

oder

Dithio-Phosphat-Rückgrat).

Thio-Phosphat-

Wesentliches Merkmal einer Nukleinsäure im Sinne der vorliegenden Erfindung ist, daß sie natürlich vorkommende cDNA oder RNA sequenzspezifisch binden kann.

Nukleinsäure-Oligomer Nukleinsäure nicht näher spezifizierter Basenlänge (z. B. Nukleinsäure-Oktamer: eine Nukleinsäure mit beliebigem Rückgrat, bei dem 8 Pyrimidin- oder Purin-Basen kovalent aneinander gebunden sind).

Oligomer

Äquivalent zu Nukleinsäure-Oligomer.

Oligonukleotid

Äquivalent zu Oligomer oder Nukleinsäure-Oligomer, also z. B. ein DNA, PNA oder RNA Fragment nicht näher spezifizierter Basenlänge.

Oligo

Abkürzung für Oligonukleotid.

Primer

Start-Komplementär-Fragment eines Oligonukleotids, wobei die Basenlänge des Primers nur ca. 4-8 Basen beträgt. Dient als Ansatzpunkt für die enzymatische Replikation des Oligonukleotids.

Mismatch

Zur Ausbildung der Watson Crick Struktur doppelsträngiger Oligonukleotide hybridisieren die beiden Einzelstränge derart, daß die Base A (bzw. C) des einen Strangs mit der Base T (bzw. G) des anderen Strangs Wasserstoffbrücken ausbildet (bei RNA ist T durch Uracil ersetzt). Jede andere Basenpaarung bildet keine Wasserstoffbrücken aus, verzerrt die Struktur und wird als "Mismatch" bezeichnet.

SS

single strand (Einzelstrang)

ds

double strand (Doppelstrang)

Photoinduzierbar und chemisch induzierbar redoxaktive Einheiten

redoxaktive Einheit

photoinduzierbar redoxaktive Einheit oder chemisch induzierbar redoxaktive Einheit

Elektron-Donor

Der Begriff Elektron-Donor bezeichnet im Rahmen der vorliegenden Erfindung einen Bestandteil einer photoinduzierbar redoxaktiven Einheit bzw. einer chemisch induzierbar redoxaktiven Einheit. Bei einem Elektron-Donor handelt es sich um ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände ein Elektron an einen Elektron-

Akzeptor transferieren kann. Ein solcher äußerer Umstand ist z. B. die Lichtabsorption durch den Elektron-Donor oder -Akzeptor einer photoinduzierbar redoxaktiven Einheit. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der Elektron-Donor "D" an den/einen Elektron-Akzeptor "A" ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D*A- aus oxidiertem Donor und reduziertem Akzeptor. Ein weiterer solcher äußerer Umstand kann z. B. die Oxidation oder Reduktion des Elektron-Donors oder -Akzeptors der chemisch induzierbar redoxaktiven Einheit durch ein externes Oxidations- oder Reduktionsmittel sein, also z. B. die Übertragung eines Elektrons auf den Elektron-Donor durch ein Reduktionsmittel bzw. die Abgabe eines Elektrons durch den Elektron-Akzeptor an ein Oxidationsmittel sein. Diese Oxidations- bzw. Reduktionsmittel können sowohl externe redoxaktive Substanzen sein, d. h. sie sind nicht kovalent mit der redoxaktiven Einheit, dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbunden, stehen aber mit diesen, z. B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung, in Kontakt oder sie sind kovalent mit dem Nukleinsäurewobei das Oxidations-Oligomer verbunden. Reduktionsmittel an einer Stelle des Nukleinsäure-Oligomers kovalent angebunden ist, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- oder Purin-Basen von der kovalenten Anbindungstelle der redoxaktiven Einheit entfemt ist, bevorzugt der Modifikation mit redoxaktiver Einheit entgegengesetzten Ende des Oligonukieotids in der Nähe der leitfähigen Oberfläche. Die Fähigkeit als Elektron-Donor oder -Akzeptor zu wirken ist relativ, d. h. ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände gegenüber einem anderen Molekül als Elektron-Donor wirkt, kann gegenüber diesem Molekül unter abweichenden experimentellen Bedingungen oder gegenüber einem dritten Molekül unter gleichen oder abweichenden experimentellen Bedingungen auch als Elektron-Akzeptor wirken.

Elekron-Akzeptor

Der Begriff Elektron-Akzeptor bezeichnet im Rahmen der vorliegenden Erfindung einen Bestandteil einer photoinduzierbar redoxaktiven Einheit bzw. einer chemisch induzierbar

. . .

redoxaktiven Einheit. Bei einem Elektron-Akzeptor handelt es sich um ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände ein Elektron von einem Elektron-Donor aufnehmen kann. Ein solcher äußerer Umstand ist z. B. die Lichtabsorption durch den Elektron-Donor oder -Akzeptor einer photoinduzierbar redoxaktiven Einheit. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der Elektron-Donor "D" an den/einen der Elektron-Akzeptor "A" ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D⁺A⁻ aus oxidiertem Donor und reduziertem Akzeptor. Ein weiterer solcher äußerer Umstand kann z. B. die Oxidation oder Reduktion des Elektron-Donors oder -Akzuptors der chemisch induzierbar redoxaktiven Einheit durch ein externes Oxidations- oder Reduktionsmittel sein, also z. B. die Übertragung eines Elektrons auf den Elektron-Donor durch ein Reduktionsmittel bzw. die Abgabe eines Elektrons durch den Elektron-Akzeptor an ein Oxidat ansmittel sein. Diese Oxidations- bzw. Reduktionsmittel können sowohl externe redoxaktive Substanzen sein, d. h. sie sind nicht kovalent mit der redoxaktiven Einheit, dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbunden, stehen aber mit diesen, z.B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung, in Kontakt oder sie sind kovalent mit dem Nukleinsäuredas Oxidations-Oligomer verbunden. wobei Reduktionsmittel an einer Stelle des Nukleinsäure-Oligomers kovalent angebunden ist, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- oder Purin-Basen von der kovalenten Anbindungstelle der photoinduzierbar redoxaktiven Einheit entfernt ist, bevorzugt an dem der Modifikation mit redoxaktiver Einheit entgegengesetzten Ende des Oligonukleotids in der Nähe der leitfähigen Oberfläche. Die Fähigkeit als Elektron-Akzeptor oder -Donor zu wirken ist relativ, d. h. ein Molekül, das unmittelbar oder nach Einwirkung bestimmter äußerer Umstände gegenüber einem anderen Molekül als Elektron-Akzeptor wirkt, unter abweichenden Molekül kann gegenüber diesem experimentellen Bedingungen oder gegenüber einem dritten Molekül unter gleichen oder abweichenden experimentellen Bedingungen auch als Elektron-Donor wirken.

WO 00/42217

Elektron-Donor-Molekül

Elekron-Akzeptor-Molekül

Oxidationsmittel

entspricht einem Elektron-Donor.

entspricht einem Elektron-Akzeptor.

chemische Verbindung (chemische Substanz), durch die Aufnahme von Elektronen aus einer anderen chemischen Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) oxidiert. Oxidationsmittel sich analog zu einem Elektron-Akzeptor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht unmittelbar zur photoinduzierbar redoxaktiven Einheit bzw. chemisch induzierbar redoxaktiven Einheit gehörigen Elektron-Akzeptor verwendet. Nicht unmittelbar bedeutet in diesem Zusammenhang, daß das Oxidationsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das Oxidationsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäuredie mindestens zwei kovalent verbundene Oligomers, Nukleotide oder wenigstens zwei kovalent verbundene kovalenten Purin-Basen von der oder Pyrimidin-Anbindungstelle der (photoinduzierbar) redoxaktiven Einheit entfernt ist. Insbesondere kann die Elektrode das Oxidationsmittel darstellen.

Reduktionsmittel

chemische Verbindung (chemische Substanz), die durch Abgabe von Elektronen an eine andere chemische Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) reduziert. Ein Reduktionsmittel verhält sich analog zu einem Elektron-Donor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht unmittelbar zur photoinduzierbar redoxaktiven Einheit bzw. zur chemisch induzierbar redoxaktiven Einheit gehörigen Elektron-Donor verwendet. Nicht unmittelbar bedeutet in diesem Zusammenhang, daß das Reduktionsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das

WO 00/42217

Reduktionsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäure-Oligomers, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidinoder Purin-Basen von der kovalenten Anbindungstelle der edoxaktiven Einheit entfemt ist. Insbesondere kann die Elektrode das Reduktionsmittel darstellen.

photoinduzierbar

photoinduzierbar bedeutet, daß eine gewisse Eigenschaft erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entfaltet wird. So entfaltet z. B. eine photoinduzierbar redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, unter bestimmten äußeren Umständen innerhalb der photoinduzierbar redoxaktiven Einheit eine Ladungstrennung durchzuführen, also z. B. den Zustand D⁺A-auszubilden, und an ein anderes geeignetes Oxidationsmittel Elektronen abzugeben oder von einem anderen geeigneten Reduktionsmittel Elektronen aufzunehmen, erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge. Ein weiteres Beispiel ist die photoinduzierbar reaktive Gruppe, d. h. eine Gruppe, die erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge reaktiv wird.

redoxaktiv

redoxaktiv bezeichnet die Eigenschaft einer redoxaktiven Einheit unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben oder von einem geeigneten Reduktionsmittel Elektronen aufzunehmen bzw. die Eigenschaft einer redoxaktiven Substanz unter bestimmten äußeren Umständen an einen geeigneten Elektron-Akzeptor Elektronen abzugeben oder von einem geeigneten Elektron-Donor Elektronen aufzunehmen.

freie, redoxaktive Substanz freies, <u>nicht kovalent mit der redoxaktiven</u> Einheit, <u>dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbundes,</u> aber mit diesen, <u>z. B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung,</u> in Kontakt <u>stehendes_Oxidationsoder Reduktionsmittel</u>, wobei die freie redoxaktive Substanz z. B. ein ungeladenes Molekül, eine beliebiges Salz oder ein redoxaktives Protein oder Enzym (Oxydoreductase) sein kann. Die freie redoxaktive Substanz ist dadurch gekennzeichnet, daß sie den oxidierten Donor (bzw. den

photoinduzierbar redoxaktive Einheit reduzierten Akzeptor) der photoinduzierbar redoxaktive Einheit re-reduzieren (bzw. re-oxidieren) kann bzw. daß die freie, redoxaktive Substanz den Donor (bzw. den Akzeptor) der chemisch induzierbar redoxaktiven Einheit reduzieren (bzw. oxidieren) kann.

Oberbegriff für eine Einheit, die ein oder mehrere Elektron-Donor-Moleküle und ein oder mehrere Elektron-Akzeptor-Moleküle enthält, wobei dieses (diese) Elektron-Donor-Molekül(e) und/oder dieses (diese) Elektron-Akzeptor-Molekül(e) in ein oder mehrere Makromoleküle eingebettet sein können. Elektron-Donor(en) und Elektron-Akzeptor(en) können untereinander durch eine oder mehrere kovalente Bindungen, durch Wasserstoff-Brückenoder ionische van-der-Waals-Brücken, durch Bindungen, Koordination mittels durch oder Wechselwirkung -Akzeptation miteinander Elektronenpaar-Donation und verbunden sein, wobei kovalente Verbindungen direkte oder indirekte (z. B. über einen Spacer, nicht aber über ein Nukleinsäure-Oligomer) Verbindungen sein können. Daneben Elektron-Elektron-Donor(en) und/oder können die Akzeptor(en), falls sie in ein oder mehrere Makromolekül(e) eingebettet sind, mit dem (den) Makromolekül(en) durch kovalente Anbindung an das (die) Makromolekül(e), durch Kavitäten passende molekulare Einkapseln in (Bindungstaschen) des Makromoleküls (der Makromoleküle), durch ionische Bindungen, Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, π-π-Wechselwirkung oder durch Elektronenpaar-Donation und mittels Koordination Akzeptation zwischen dem(n) Makromolekül(en) und dem(n) dem(n) Elektron-Elektron-Donor-Molekül(en) und/oder verbunden sein. Sind mehrere Akzeptor-Molekül(en) Makromoleküle Bestandteil der photoinduzierbar redoxaktiven Einheit kann die Bindung der Makromoleküle untereinander ebenfalls kovalent, ionisch, durch Wasserstoff-Brücken- π - π -Wechselwirkung van-der-Waals-Brücken, Bindungen, oder durch Koordination mittels Elektronenpaar-Donation und Wesentliche Merkmale der erfolgen. -Akzeptation photoinduzierbar redoxaktiven Einheit sind neben der Zusammensetzung aus Elektron-Donor(en) und Elektron-

Akzeptor(en) oder aus Elektron-Donor(en) und Elektron-Akzeptor(en) und Makromolekül(en): (i) die Einheit ist in den erfindungsrelevanten Erscheinungsformen (Elektron-Donor(en) und Elektron-Akzeptor(en) im ursprünglichen bzw. oxidierten oder reduzierten Zustand) stabil und dissoziiert nicht in ihre Bestandteile, (ii) die Einheit enthält keine Nukleinsäure, (iii) die Zusammensetzung der Einheit aus Elektron-Donor(en) und Elektron-Akzeptor(en) oder aus Elektron-Donor(en) und Elektron-Akzeptor(en) und Makromolekül(en) kann - unabhängig von der Bindung zwischen den Bestandteilen - vom Fachmann erkannt werden, da sie prinzipiell auch als Einzelmoleküle vorkommen können Elektron-Donor(en) und (iv) und Akzeptor(en) der photoinduzierbar redoxaktiven Einheit wirken unter gleichen oder ähnlichen äußeren Umständen in Form von Einzelmolekülen in Lösung als Elektron-Donor(en) und Elektron-Akzeptor(en), d. h. auch bei freien gelösten Elektron-Donor(en) Elektron-Akzeptor(en) kann und unmittelbar oder nach Einwirkung bestimmter äußerer Umstände, entsprechend den Umständen die innerhalb der photoinduzierbar redoxaktiven Einheit zu einem Elektrontransfer führen, ein Elektron vom (von den) gelösten Elektron-Donor(en) auf den (die) übertragen werden. Die gelösten Elektron-Akzeptor(en) photoinduzierbar redoxaktive Einheit kann z. B. jedes beliebige photoinduzierbar redoxaktive Protein/Enzym oder jeder beliebige photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der/ein Elektron-Donor an einen der Elektron-Akzeptoren ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D*A- aus einem oxidierten Donor und einem reduzierten Akzeptor. Dieser Vorgang innerhalb der photoinduzierbar redoxaktiven Einheit wird als photoinduzierte Ladungstrennung bezeichnet. Bei entsprechend gewählten äußeren Umständen entfaltet die photoinduzierbar redoxaktive Einheit ihre Redoxaktivität, also Oxidationsmittel ihre Eigenschaft, an einen geeignetes geeigneten abzugeben oder von einem Elektronen erst im aufzunehmen, Elektronen Reduktionsmittel

ladungsgetrennten Zustand, da das Reduktionsmittel (bzw. Oxidationsmittel) nur auf den oxidierten Donor (bzw. vom reduzierten Akzeptor) der photoinduzierbar redoxaktiven Einheit Elektronen überträgt (bzw. aufnimmt), z. B. in Gegenwart eines Reduktionsmittels, das D⁺, jedoch nicht D, reduzieren kann (bzw. in Gegenwart eines Oxidationsmittels das A⁻, jedoch nicht A, oxidieren kann). Insbesondere kann dieses Oxidations- bzw. Reduktionsmittel auch eine Elektrode sein, wobei die photoinduzierbar redoxaktive Einheit erst nach der photoinduzierten Ladungstrennung ein Elektron an eine Elektrode abgeben (bzw. von dieser aufnehmen) kann, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A⁻, jedoch nicht A, oxidiert (bzw. D⁺, jedoch nicht D, reduziert) wird.

chemisch induzierbar redoxaktive Einheit

entspricht in Zusammensetzung und Funktionsweise einer photoinduzierbar redoxaktive Einheit, wobei aber im Unterschied zur Funktionsweise einer photoinduzierbar redoxaktiven Einheit Photoaktivierung als äußerer Umstand zur Entfaltung der Redoxaktivität der redoxaktive Einheit ausgeschlossen ist. Die redoxaktive Einheit kann also z. B. jedes beliebige redoxaktive Protein/Enzym oder jeder beliebige redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Bei entsprechend gewählten äußeren Umständen entfaltet die redoxaktive Einheit Redoxaktivität, also ihre Eigenschaft, z. B. an ein geeignetes Oxidationsmittel Elektronen abzugeben, erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D", der nur im reduzierten Zustand "D" " ein Elektron auf den Akzeptor "A" übertragen kann und das Oxidationsmittel nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einheit Elektronen aufnimmt, also in Gegenwart eines Oxidationsmittels, das A, jedoch nicht A, oxidieren kann (sukzessive Ladungsübertragung). Insbesondere kann dieses Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann bei abweichend gewählten äußeren Umständen die redoxaktive Einheit ihre Redoxaktivität, also z. B. ihre Eigenschaft von einem geeigneten Reduktionsmittel



Elektronen aufzunehmen, erst nach Übertragung eines von einem Elektron-Akzeptor "A" Elektrons Oxidationsmittel entfalten, wenn nur der oxidierte Akzeptor "A* " ein Elektron vom Donor D aufnehmen kann und das Reduktionsmittel nur auf den oxidierten Donor "D" der redoxaktiven Einheit Elektronen übertragen kann, z. B. in Gegenwart eines Reduktionsmittels, das D+, jedoch nicht D, (sukzessive Ladungsübertragung). reduziert kann Insbesondere kann dieses Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D⁺, jedoch nicht D, reduziert wird.

photoinduzierbar redoxaktives Protein/Enzym

besteht in der Regel aus sogenanntem Apoprotein, dem (den) bevorzugten Makromolekül(en) der vorliegenden Erfindung, und Cofaktoren, den Elektron-Donor(en) und Elektron-Akzeptor(en) im Sinne der vorliegenden Erfindung. Die Ladungstrennung innerhalb des photoinduzierte photoaktivierbar redoxaktiven Proteins/Enzyms wird durch Licht bestimmter oder beliebiger Wellenlänge ausgelöst. So sind zum Beispiel im photosynthetischen Reaktionszentrum (reaction center, RC) als Cofaktoren ein primärer Elektron-Donor P und mehrere verschiedene Elektron-Akzeptoren A, darunter auch Quinon-Cofaktor(en) Q, in eine Proteinmatrix eingebettet und bilden so eine "polymolekulare" Einheit (vgl. Struktur 1). Die Einbettung erfolgt in diesem Fall durch Einkapselung der Cofaktoren in passende Kavitäten, sogennante Bindungstaschen der Proteinmatrix aus mehreren Protein-Untereinheiten. Sowohl die Protein-Untereinheiten als auch die Einkapselung der Cofaktoren in die Proteinmatrix ist im Fall einiger natürlich vorkommender RC durch nicht-Lichteinstrahlung Bei realisiert. kovalente Bindungen geeigneter Wellenlänge gibt der primäre Donor ein Elektron an einen der Elektron-Akzeptoren ab und es bildet sich, zumindest temporär, aus den anfänglich neutralen Cofaktoren ein ladungsgetrennter RC-Zustand P*A-, insbesondere auch der Zustand P⁺Q⁻.

Enzym

chemisch induzierbar entspricht in Zusammensetzung und Funktionsweise einem redoxaktives Protein/ photoinduzierbar redoxaktiven Protein/Enzym, wobei aber im photoinduzierbar einer Funktionsweise zur Unterschied

photoinduzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex redoxaktiven Einheit Photoaktivierung als äußerer Umstand zur Einheit Entfaltung der Redoxaktivität der redoxaktive ausgeschlossen ist; das chemisch induzierbar redoxaktive Protein/Enzym besteht in der Regel aus sogenanntem Apoprotein, dem (den) bevorzugten Makromolekül(en) der vorliegenden Erfindung, und Cofaktoren, den Elektron-Elektron-Akzeptor(en) im Sinne und Donor(en) vorliegenden Erfindung. Die Eigenschaft des redoxaktiven Proteins/Enzyms zur sukzessiven Ladungsübertragung wird durch eine freie redoxaktive Substanz (Substrat) ausgelöst.

Verbindung aus einem oder mehreren Elektron-Donor Molekülen D1, D2, D3 etc. und mindestens einem oder mehreren geeigneten Elektron-Akzeptor Molekülen A1, A2, A3 etc., wobei die Elektron-Donor(en) und Elektron-Akzeptor(en) untereinander durch eine oder mehrere kovalente oder ionische Bindungen, durch Wasserstoff-Erücken-Bindungen, van-der-Waals-Brücken, durch π - π mittels Koordination Wechselwirkung oder durch und -Akzeptation miteinander Elektronenpaar-Donation verbunden sind. Kovalente Verbindungen in diesem Sinne können direkte oder indirekte (z. B. über einen Spacer, nicht aber über ein Nukleinsäure-Oligomer) Verbindungen sein. Wesentliche Merkmale des photoinduzierbar redoxaktiven, bimolekularen Elekton-Donorwenigstens verknüpften, /Elektron-Akzeptor-Komplexes sind neben Zusammensetzung aus Elektron-Donor(en) und Elektron-Elekton-Donor-/Elektron-Akzeptor-Akzeptor(en): (i) der Komplex ist in den erfindungsrelevanten Erscheinungsformen (Elektron-Donor(en) und Elektron-Akzeptor(en) ursprünglichen bzw. oxidierten oder reduzierten Zustand) stabil und dissoziiert nicht in seine Bestandteile, (ii) die Einheit enthält keine Nukleinsäure, (iii) die Zusammensetzung des wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes aus Elektron-Donor(en) und Elektron-Akzeptor(en) kann - unabhängig von der Bindung zwischen den Bestandteilen - vom Fachmann erkannt werden und (iv) Elektron-Akzeptor(en) des und Elektron-Donor(en) photoinduzierbar redoxaktiven. verknüpften, wenigstens Elekton-Donor-/Elektron-Akzeptor-Komplex bimolekularen

wirken unter gleichen oder ähnlichen äußeren Umständen auch in Form von Einzelmolekülen in Lösung als Elektron-Donor(en) und Elektron-Akzeptor(en), d. h. auch bei freien gelösten Elektron-Donor(en) und Elektron-Akzeptor(en) kann unmittelbar oder nach Einwirkung bestimmter äußerer Umstände, entsprechend den Umständen die innerhalb der photoinduzierbar redoxaktiven Einheit zu einem Elektrontransfer führen, ein Elektron vom (von den) gelösten Elektron-Donor(en) auf den (die) übertragen werden. gelösten Elektron-Akzeptor(en) verknüpfte, wenigstens photoinduzierbar redoxaktive. Elekton-Donor-/Elektron-Akzeptor-Komplex bimolekulare entspricht in seiner erfindungsrelevanten Funktionsweise einem photoinduzierbar redoxaktiven Protein/Enzym, d. h. auch hier kommt es durch Lichteinstrahlung geeigneter Wellenlänge zur photoinduzierten Ladungstrennung und es wird, zumindest temporär, ein ladungsgetrennter Zustand D*A- gebildet (wobei D für ein beliebiges D1, D2, D3 etc. und A für ein beliebiges A1, A2, A3 etc. steht). Im Ausdruck verknüpfter, wenigstens redoxaktiver, "photoinduzierbar bimolekularer Elekton-Donor-/ Elektron-Akzeptor-Komplex" steht der Begriff "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn der Donor mit dem Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden ist.

chemisch induzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex

entspricht in Zusammensetzung und Funktionsweise einem verknüpften, wenigstens redoxaktiven, photoinduzierbar Elektron-Donor/Elektron-Akzeptor-Komplex, bimolekularen wobei aber im Unterschied zur Funktionsweise eines wenigstens verknüpften, redoxaktiven, photoinduzierbar bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes Photoaktivierung als äußerer Umstand zur Entfaltung der Redoxaktivität der redoxaktiven Einheit ausgeschlossen ist. Bei entsprechend gewählten äußeren Umständen entfaltet die redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, B. an ein geeignetes Oxidationsmittel Elektronen abzugeben, erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D", der nur im reduzierten Zustand "D-" ein Elektron auf den Akzeptor "A"



übertragen kann und das Oxidationsmittel nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einheit Elektronen aufnimmt, also in Gegenwart eines Oxidationsmittels das A-, kann (sukzessive oxidieren nicht A. iedoch kann dieses Insbesondere Ladungsübertragung). Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann bei abweichend gewählten äußeren Umständen die redoxaktive Einheit ihre Redoxaktivität, also z. B. ihre Eigenschaft von einem geeigneten Reduktionsmittel Elektronen aufzunehmen, erst nach Übertragung eines Elektrons von einem Elektron-Akzeptor "A" auf ein Oxidationsmittel entfalten, wenn nur der oxidierte Akzeptor "A* " ein Elektron vom Donor D aufnehmen kann und das Reduktionsmittel nur auf den oxidierten Donor "D" der redoxaktiven Einheit Elektronen übertragen kann, z. B. in Gegenwart eines Reduktionsmittels, das D*, jedoch nicht D. reduzieren kann (sukzessive Ladungsübertragung). Insbesondere kann dieses Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D+, jedoch nicht D, reduziert wird. Im verknüpfter. "redoxaktiver. Ausdruck Elekton-Donor-/Elektron-Akzeptor-Komplex" bimolekularer steht der Begriff "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn der Donor mit dem Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden ist.

RC

photoinduzierbar Reaktionszentrum. Beispiel eines redoxaktiven Proteins/Enzyms. Bei dem Protein/Enzym handelt es sich um einen sogenannten Pigment/Proteinmehreren Komplex der aus Apoprotein mit Proteinuntereinheiten und mehreren Cofaktoren (im Beispiel In solchen Pigmente) handelt. RC sogenannte Pigment/Protein-Komplexen spielen sich die ersten Schritte der lichtgetriebenen Ladungstrennung der bakteriellen oder pflanzlichen Photosynthese ab. Das RC der Photosynthese Rhodobacter betreibenden Stammes Bakterien des sphaeroides z. B. (vgl. Struktur 1) besteht aus drei Protein-

Untereinheiten und acht Cofaktoren (Pigmenten). Die Cofaktoren. Bakteriochlorophyll-Dimer ein zwei Bakteriochlorophyll-Monomere B_A und B_B, zwei Bakteriopheophytin-Monomere H_A und H_{B} und zwei Ubichinon-50 (UQ) Moleküle Q_A und Q_B, sind in den jeweiligen Protein-Bindungstaschen (also der P-, B_Aetc. Bindungstasche) lokalisiert.

Q_A-Protein-Bindungstasche Proteinbindungstasche bzw. Proteinumgebung, in der sich der Chinon-Cofaktor Q_A befindet. In RC von z.B. *Rhodobacter sphaeroides* ist der Chinon-Cofaktor Q_A ein Ubichinon-50 (vgl. Struktur 1).

Q_A-Bindungstasche

Q_A-Protein-Bindungstasche

Chemische Substanzen/Gruppen

ZnBChl Zn-Bakteriochlorophyll (Formel 11 mit M = Zn)

Q allgemein für Chinon (engl. Quinone), im Beispiel 3 und den sich darauf beziehenden Textpassagen ist Q ein modifiziertes

daraur beziehenden rexipassagen ist die im men

Anthrachinon oder Pyrrollochinolinochinon (PQQ).

UQ Ubichinon-50, RC-Cofaktor und temporärer Elektron-Akzeptor

z. B. im RC der Photosynthese betreibenden Bakterien aus z.B. Rhodobacter sphaeroides oder Rhodopseudomonas

viridis.

(cyt c₂)²⁺ reduzierte Form des Cytochrom c₂, ein frei bewegliches Häm-

Protein, das in der bakteriellen Photosynthese in *Rhodobacter* sphaeroides den oxidierten primären Donor P⁺ zu P reduziert;

Beispiel einer redoxaktiven Substanz.

PQQ Pyrrolo-Chinolino-Chinon, entspricht: 4,5-Dihydro-4,5-dioxo-

1H-pyrrolo-[2,3-f]-chinolin-2,7,9-tricarboxylsäure)

EDTA Ethylendiamin-Tetraacetat (Natriumsalz)

sulfo-NHS N-Hydroxysulfosuccinimid

EDC (3-Dimethylaminopropyl)-carbodiimid

HEPES N-[2-Hydroxyethyl]piperazin-N'-[2-ethansulfonsäure]

Tris Tris-(hydroxymethyl)-aminomethan

Alkyl

aesättiate eine bezeichnet "Alkyl" Der Beariff Kohlenwasserstoffgruppe, die geradkettig oder verzweigt ist (z.B. Ethyl, 2,5-Dimethylhexyl oder Isopropyl etc.). Wenn "Alkyl" benutzt wird, um auf einen Linker oder Spacer zu verweisen, bezeichnet der Begriff eine Gruppe mit zwei verfügbaren Valenzen für die -CH₂CH₂-, (z. B. Verknüpfung kovalente -CH₂CH₂CH₂--CH₂C(CH₃)₂CH₂CH₂C(CH₃)₂CH₂oder Bevorzugte Alkylgruppen als Substituenten oder Seitenketten R sind solche der Kettenlänge 1 - 30 (längste durchgehende Kette von kovalent aneinander gebundenen Atomen). Bevorzugte Alkylgruppen als Linker oder Spacer sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge hier die kürzeste durchgehende Verbindung zwischen den durch den Linker oder Spacer verbundenen Strukturen, also zwischen den zwei Molekülen bzw. zwischen Oberflächenmolekül oder einer einem Oberflächenatom. Oberflächenmolekülgruppe und einem anderen Molekül, darstellt.

Alkenyl

Alkylgruppen, bei denen eine oder mehrere der C-C Einfachbindungen durch C=C Doppelbindungen ersetzt sind.

Alkinyl

Alkyl- oder Alkenylgruppen, bei denen eine oder mehrere der C-C Einfach- oder C=C Doppelbindungen durch C=C Dreifachbindungen ersetzt sind.

Hetero-Alkyl

Alkylgruppen, bei denen eine oder mehrere der C-H Bindungen oder C-C Einfachbindungen durch C-N, C=N, C-P, C=P, C-O, C=O, C-S oder C=S Bindungen ersetzt sind.

Hetero-Alkenyl

Alkenylgruppen, bei denen eine oder mehrere C-H Bindungen, C-C Einfach- oder C=C Doppelbindungen durch C-N, C=N, C-P, C=P, C-C, C=O, C-S oder C=S Bindungen ersetzt sind.

Hetero-Alkinyl

Alkinylgruppen, bei denen eine oder mehrere der C-H Bindungen, C-C Einfach-, C=C Doppel- oder C≡C Dreifachbindung durch C-N, C=N, C-P, C=P, C-O, C=O, C-S oder C=S Bindungen ersetzt sind.

Linker

molekulare Verbindung zwischen zwei Molekülen bzw. zwischen einem Oberflächenatom, Oberflächenmolekül oder einer Oberflächenmolekülgruppe und einem anderen Molekül.

In der Regel sind Linker als Alkyl-, Alkenyl-, Alkinyl-, Hetero-Alkyl-, Hetero-Alkenyl- oder Heteroalkinylkette käuflich zu erwerben, wobei die Kette an zwei Stellen mit (gleichen oder verschiedenen) reaktiven Gruppen derivatisiert ist. Diese chemischen einfachen/bekannten Gruppen bilden in Reaktionen mit den entsprechenden Reaktionspartner eine kovalente chemische Bindung aus. Die reaktiven Gruppen können auch photoaktivierbar sein, d. h. die reaktiven Gruppen werden erst durch Licht bestimmter oder beliebiger Wellenlänge aktiviert. Bevorzugte Linker sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge hier die kürzeste durchgehende Verbindung zwischen den zu verbindenden Strukturen, also zwischen den zwei Molekülen bzw. zwischen einem oder einer Oberflächenatom. Oberflächenmolekül Oberflächenmolekülgruppe und einem anderen Molekül, darstellt.

Spacer

Linker, der über die reaktiven Gruppen an eine oder beide der zu verbindenden Strukturen (siehe Linker) kovalent angebunden ist. Bevorzugte Spacer sind solche der Kettenlänge 1 - 20, insbesondere der Kettenlänge 1 - 14, wobei die Kettenlänge die kürzeste durchgehende Verbindung zwischen den zu verbindenden Strukturen darstellt.

(n x HS-Spacer)oligo

Nukleinsäure-Oligomer, an das n Thiolfunktionen über jeweils einen Spacer angebunden sind, wobei die Spacer jeweils eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Thiolfunktion und Nukleinsäure-Oligomer) aufweisen können, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14. Diese Spacer können wiederum an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diesem durch Modifikation angebrachte reaktive Gruppen gebunden sein und "n" ist eine beliebige ganze Zahl, insbesondere eine Zahl zwischen 1 und 20.

(n x R-S-S-Spacer)oligo

Nukleinsäure-Oligomer, an das n Disulfidfunktionen über jeweils einen Spacer angebunden sind, wobei ein beliebiger Rest R die Disulfidfunktion absättigt. Der Spacer zur Anbindung der Disulfidfunktion an das Nukleinsäure-Oligomer

kann jeweils eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Disulfidfunktion und Nukleinsäure-Oligomer) aufweisen, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14. Diese Spacer können wiederum kann an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diesem durch Modifikation angebrachte reaktive Gruppen gebunden sein. Der Platzhalter n ist eine beliebige ganze Zahl, insbesondere eine Zahl zwischen 1 und 20.

oligo-Spacer-S-S-Spacer-oligo

zwei gleiche oder verschiedene Nukleinsäure-Oligomere, die über eine Disulfid-Brücke miteinander verbunden sind, wobei die Disulfidbrücke über zwei beliebige Spacer an die Nukleinsäure-Oligomere angebunden ist und die beiden Spacer eine unterschiedliche Kettenlänge (kürzeste durchgehende Verbindung zwischen Disulfidbrücke und dem jeweiligen Nukleinsäure-Oligomer) aufweisen können, insbesondere jeweils eine beliebige Kettenlänge zwischen 1 und 14 und diese Spacer wiederum an verschiedene natürlich am Nukleinsäure-Oligomer vorhandene oder an diese durch Modifikation angebrachte reaktive Gruppen gebunden sein können.

Modifizierte Oberflächen/Elektroden

Mica

Muskovit-Plättchen, Trägermaterial zum Aufbringen dünner Schichten.

Au-S-(CH₂)₂-ss-oligo-Spacer-UQ(RC) Gold-Film auf Mica mit kovalent aufgebrachter Monolayer aus derivatisiertem 12Bp Einzelstrang DNA-Oligonukleotid (Sequenz: TAGTCGGAAGCA). Hierbei ist die endständige Phosphatgruppe des Oligonukleotids am 3' Ende mit (HO-(CH₂)₂-S)₂ zum P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert, wobei die S-S Bindung homolytisch gespalten wird und je eine Au-S-R Bindung bewirkt. Die endständige Base Thymin am 5'- Ende des Oligonukleotids ist am C-5 Kohlenstoff mit -CH=CH-CO-NH-CH₂-CH₂-NH₂ modifiziert, wobei dieser Rest wiederum über seine freie Aminogruppe durch Amidbildung mit der Carbonsäuregruppe des modifizierten Ubichinon-50 verbunden ist. Anschließend wird das UQ mit dem restlichen RC rekonstituiert.

Au-S-(CH₂)₂-ds-oligo-

| Au-S-(CH₂)₂-ss-oligo-Spacer-UQ(RC) hybridisiert mit dem zu ss-

Spacer-UQ(RC)

oligo (Sequenz: TAGTCGGAAGCA) komplementären Oligonukleotid.

Au-S-(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl identisch zu *Au-S-(CH₂)₂-ss-oligo-Spacer-UQ(RC)* mit der Ausnahme, daß, statt des über UQ angebundenen RCs, Q-ZnBChI als photoinduzierbar redoxaktive Einheit angebunden ist.

Au-S-(CH₂)₂-ds-oligo-Spacer-Q-ZnBChI Au-S-(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl hybridisiert mit dem zu ssoligo (Sequenz: TAGTCGGAAGCA) komplementären Oligonukleotid.

Elektrochemie

Elektrodenpotential, das an der Arbeitselektrode anliegt.

Potential beim Strom-Maximum der Oxidation einer reversiblen Elektrooxidation oder -reduktion.

Stromdichte (Strom pro cm² Elektrodenoberfläche)

Cyclovoltametrie A

Aufzeichnung einer Strom/Spannungskurve. Hierbei wird das Potential einer stationären Arbeitselektrode zeitabhängig linear verändert, ausgehend von einem Potential, bei dem keine Elektrooxidation oder -reduktion stattfindet bis zu einem Potential, bei dem eine gelöste oder an die Elektrode adsorbierte Spezies oxidiert oder reduziert wird (also Strom fließt). Nach Durchlaufen des Oxidations- bzw. Reduktionsvorgangs, der in der Strom/Spannungskurve einen zunächst ansteigenden Strom und nach Erreichen eines Maximums einen allmählich abfallenden Strom erzeugt, wird die Richtung des Potentialvorschubs umgekehrt. Im Rücklauf wird dann das Verhalten der Produkte der Elektrooxidation oder -reduktion aufgezeichnet.

Amperometrie

Aufzeichnung einer Strom/Zeitkurve. Hierbei wird das Potential einer stationären Arbeitselektrode z. B. durch einen Potentialsprung auf ein Potential gesetzt, bei dem die Elektrooxidation oder -reduktion einer gelösten oder adsorbierten Spezies stattfindet und der fließende Strom wird in Abhängigkeit von der Zeit aufgezeichnet.

Die vorliegende Erfindung betrifft ein Nukleinsäure-Oligomer, das durch chemische Bindung einer redoxaktiven Einheit modifiziert ist. Die redoxaktive Einheit ist entweder eine photoinduzierbar redoxaktive Einheit oder eine chemisch induzierbar redoxaktive Einheit. Die photoinduzierbar redoxaktive Einheit kann nach

photoinduzierter Abgabe eines Elektrons an ein externes Oxidationsmittels, z. B. einer Elektrode, oder Aufnahme eines Elektrons von einem externen Reduktionsmittel, z. B. einer Elektrode, durch eine freie redoxaktive Substanz rereduziert bzw. re-oxidiert, also in seinen ursprünglichen Zustand zurückversetzt werden. Die chemisch induziert redoxaktive Einheit kann nach Abgabe eines Elektrons an ein externes Oxidationsmittels von einem externen Reduktionsmittel, z. B. einer Elektrode, reduziert oder nach Aufnahme eines Elektrons von einem externen Reduktionsmittel durch ein externes Oxidationsmittel, z. B. einer Elektrode, oxidiert werden.

Als Nukleinsäure-Oligomer wird im Rahmen der vorliegenden Erfindung eine Verbindung aus wenigstens zwei kovalent verbundenen Nukleotiden oder aus wenigstens zwei kovalent verbundenen Pyrimidin- (z. B. Cytosin, Thymin oder Uracil) oder Purin-Basen (z. B. Adenin oder Guanin), bevorzugt ein DNA-, RNA- oder PNA-Fragment, verwendet. In der vorliegenden Erfindung bezieht sich der Begriff Nukleinsäure auf ein beliebiges "Rückgrat" der kovalent verbundenen Pyrimidin- oder Purin-Basen, wie z. B. auf das Zucker-Phosphat Fuckgrat der DNA, cDNA oder RNA, auf ein Peptid-Rückgrat der PNA oder auf analoge Rückgrat-Strukturen, wie z. B. ein Thio-Phosphat-, ein Dithio-Phosphat- oder ein Phosphoramid-Rückgrat. Wesentliches Merkmal einer Nukleinsäure im Sinne der vorliegenden Erfindung ist, daß sie natürlich vorkommende cDNA oder RNA sequenzspezifisch binden kann. Alternativ zu dem Begriff "Nukleinsäure-Oligomer" werden die Begriffe "(Sonden-) Oligonukleotid", "Nukleinsäure" oder "Oligomer" verwendet.

Der Begriff "Elektron-Akzeptor" bzw. "Elektron-Akzeptor-Molekül" und der Begriff "Elektron-Donor" bzw. "Elektron-Donor-Molekül" bezeichnet im Rahmen der vorliegenden Erfindung einen Bestandteil einer redoxaktiven Einheit.

Unter einer "redoxaktiven Einheit" wird im Rahmen der vorliegenden Erfindung jede Einheit verstanden, die einen oder mehrere Elektron-Donor-Moleküle und einen oder mehrere Elektron-Akzeptor-Moleküle enthält. Die Elektron-Donor-Molekül(e) oder -Molekülteil(e) und Elektron-Akzeptor-Molekül(e) oder -Molekülteil(e) dieser redoxaktiven Einheit können untereinander durch eine oder mehrere kovalente oder ionische Bindungen, durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, durch π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation miteinander verbunden sein, wobei kovalente Bindungen direkte oder indirekte (z. B. über einen Spacer, nicht aber über ein Nukleinsäure-Oligomer) Bindungen sein können. Außerdem können die Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-Molekül(e) in ein oder mehrere Makromolekül(e)

eingebunden sein, wobei diese Einbindung durch Einkapseln in passende molekulare Kavitäten (Bindungstaschen) des Makromoleküls (der Makromoleküle), durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation zwischen dem(n) Makromolekül(en) und dem(n) Elektron-Donor-Molekül(en) und/oder dem(n) Elektron-Akzeptor-Molekül(en) erfolgt. In diesem Fall bilden also die Makromolekül(e) und die Elektron-Donor-Molekül(e) und die Elektron-Akzeptor-Molekül(e) die redoxaktive Einheit. Sind mehrere Makromoleküle Bestandteil der redoxaktiven Einheit kann die Bindung der Makromoleküle untereinander ebenfalls kovalent, ionisch, durch Wasserstoff-Brücken-Bindungen, van-der-Waals-Brücken, π - π -Wechselwirkung oder durch Koordination mittels Elektronenpaar-Donation und -Akzeptation erfolgen.

Die angesprochenen Donor- und Akzeptor-Moleküle bilden erfindungsgemäß eine redoxaktive Einheit, d. h. sie sind direkt oder über weitere Molekülteile aneinander gebunden. Einzige erfindungsgemäße Einschränkung der die Bestandteile der redoxaktiven Einheit verbindenden Moleküle oder Molekülteile ist der Ausschluß von Nukleinsäure-Oligomeren. Gemäß der vorliegenden Erfindung ist die redoxaktive Einheit als eine komplette Einheit an das Sonden-Oligonukleotid gebunden, wobei natürlich mehrere chemische Bindungen zwischen Oligonukleotid und der redoxaktiven Einheit ausgebildet werden können. Durch den Ausschluß von Nukleinsäure-Oligomeren als die die Bestandteile der redoxaktiven Einheit verbindenden Moleküle oder Molekülteile soll verdeutlicht werden, daß nicht einzelne Teile der redoxaktiven Einheit an verschiedenen Stellen des Sonden-Oligonukleotids angebunden sind. Das Sonden-Oligonukleotid stellt also explizit nicht die Verbindung zwischen den Elektron-Donor-Molekül(en) oder -Molekülteil(en) und den Elektron-Akzeptor-Molekül(en) oder -Molekülteil(en) der redoxaktiven Einheit dar.

Die redoxaktive Einheit ist entweder eine photoinduzierbar redoxaktive Einheit oder eine chemisch induzierbar redoxaktive Einheit.

"Photoinduzierbar" heißt im Rahmen der vorliegenden Erfindung, daß die Redoxaktivität der photoinduzierbar redoxaktiven Einheit, also deren Eigenschaft unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben oder von einem geeigneten Reduktionsmittel Elektronen aufzunehmen, erst durch Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entfaltet wird. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der Elektron-Donor "D" an einen der Elektron-Akzeptoren "A" ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand

D*A⁻ aus oxidiertem Donor und reduziertem Akzeptor. Dieser Vorgang innerhalb der photoinduzierbar redoxaktiven Einheit wird als photoinduzierte Ladungstrennung bezeichnet. Bei entsprechend gewählten äußeren Umständen entfaltet die photoinduzierbar redoxaktive Einheit ihre Redoxaktivität erst im ladungsgetrennten Zustand, da das Reduktionsmittel (bzw. das Oxidationsmittel) nur auf den oxidierten Donor (bzw. vom reduzierten Akzeptor) der photoinduzierbar redoxaktiven Einheit Elektronen übertragen kann (bzw. aufnehmen kann), z. B. in Gegenwart eines Oxidationsmittels, das A⁻, jedoch nicht A, oxidieren kann (bzw. in Gegenwart eines Reduktionsmittels, das D*, jedoch nicht D, reduzieren kann).

Insbesondere kann das angesprochene Oxidations- bzw. Reduktionsmittel eine Elektrode sein, wobei die photoinduzierbar redoxaktive Einheit erst nach der photoinduzierten Ladungstrennung ein Elektron an die Elektrode abgeben (bzw. von dieser aufnehmen) kann, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert (bzw. D+, jedoch nicht D, reduziert) wird. Daneben kann das Oxidations- bzw. Reduktionsmittel eine freie, redoxaktive Substanz sein, wobei die photoinduzierbar redoxaktive Einheit erst nach der photoinduzierten Ladungstrennung ein Elektron an die freie, redoxaktive Substanz abgeben (bzw. von dieser aufnehmen) kann, z. B. wenn die freie redoxaktive Substanz A-, jedoch nicht A, oxidiert (bzw. D+, jedoch nicht D, reduziert).

"Chemisch induzierbar" heißt im Rahmen der vorliegenden Erfindung, daß die Redoxaktivität der chemisch induzierbar redoxaktiven Einheit eine redoxaktive Einheit verstanden, also deren Eigenschaft unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben (bzw. von einem geeigneten Reduktionsmittel Elektronen aufzunehmen), erst nach Reduktion (bzw. nach Oxidation) durch ein externes Reduktionsmittel (bzw. Oxidatinsmittel) entfaltet wird. Die chemisch induzierbar redoxaktiven Einheit entspricht in Zusammensetzung und Funktionsweise einer photoinduzierbar redoxaktive Einheit, wobei aber im Unterschied zur Funktionsweise einer photoinduzierbar redoxaktiven Einheit Photoaktivierung als äußerer Umstand zur Entfaltung der Redoxaktivität der redoxaktive Einheit ausgeschlossen ist. Bei entsprechend gewählten äußeren Umständen entfaltet die chemisch induzierbar redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, z. B. an ein geeignetes Oxidationsmittel Elektronen abzugeben, erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D": Nur im reduzierten Zustand "D" kann der Elektron-Donor ein Elektron auf den Akzeptor "A" übertragen und das Oxidationsmittel kann nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einheit Elektronen aufnehmen, z.B. in Gegenwart eines Oxidationsmittels das A-, jedoch nicht A, oxidieren kann

(sukzessive Ladungsübertragung). Insbesondere kann das besagte Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann - bei abweichend gewählten äußeren Umständen - die chemisch induzierbar redoxaktive Einheit ihre Redoxaktivität, also z. B. ihre Eigenschaft von einem geeigneten Reduktionsmittel Elektronen aufzunehmen, erst nach Übertragung eines Elektrons von einem Elektron-Akzeptor "A" auf ein Oxidationsmittel entfalten: Nur im oxidierten Zustand "A+" kann der Elektron-Akzeptor ein Elektron vom Donor D aufnehmen und das Reduktionsmittel kann nur auf den oxidierten Donor "D+" der redoxaktiven Einheit Elektronen übertragen, z. B. in Gegenwart eines Reduktionsmittels, das D+, jedoch nicht D, reduzieren kann (sukzessive Ladungsübertragung). Insbesondere kann das besagte Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D+, jedoch nicht D, reduziert wird.

Wesentliche Merkmale der photoinduzierbar oder chemisch induzierbar redoxaktiven Einheit sind neben der Zusammensetzung aus Elektron-Donor(en) und Elektron-Akzeptor(en) oder aus @lektron-Donor(en) und Elektron-Akzeptor(en) und Makromolekül(en): (i) die Einheit ist in den erfindungsrelevanten Erscheinungsformen (Elektron-Donor(en) und Elektron-Akzeptor(en) im ursprünglichen bzw. oxidierten oder reduzierten Zustand) stabil und dissoziiert nicht in ihre Bestandteile, (ii) die Einheit enthält keine Nukleinsäure, (iii) die Zusammensetzung der Einheit aus Elektron-Donor(en) und Elektron-Akzeptor(en) oder aus Elektron-Donor(en) und Elektron-Akzeptor(en) und Makromolekül(en) kann - unabhängig von der Bindung zwischen den Bestandteilen - vom Fachmann erkannt werden, da Elektron-Donor(en) und Akzeptor(en) prinzipiell auch als Einzelmoleküle vorkommen können und (iv) Elektron-Donor(en) und Elektron-Akzeptor(en) der redoxaktiven Einheit wirken unter gleichen oder ähnlichen äußeren Umständen wie in ihrer erfindungsrelevanten Erscheinung als Bestandteile der redoxaktiven Einheit auch als Einzelmoleküle in Lösung als Elektron-Donor(en) und Elektron-Akzeptor(en), d. h. auch bei freien gelösten Elektron-Donor(en) und Elektron-Akzeptor(en) kann unmittelbar oder nach Einwirkung bestimmter äußerer Umstände, entsprechend den Umständen die innerhalb der redoxaktiven Einheit zu einem Elektrontransfer führen, ein Elektron vom (von den) gelösten Elektron-Donor(en) auf den (die) gelösten Elektron-Akzeptor(en) übertragen werden. Wie für die Elekron-Donor(en) und Elektron-Akzeptor(en) der photoinduzierbar redoxaktiven Einheit kann ein solcher äußerer Umstand für die freien, gelösten Elekron-Donor(en) und Elektron-Akzeptor(en) die Lichtabsorption durch den (die) freien, gelösten Elektron-Donor(en) oder -Akzeptor(en) sein, wobei der (ein) Elektron-Donor "D" an den (einen) Elektron-Akzeptor "A" ein Elektron abgibt und, zumindest temporär, ein ladungsgetrennter Zustand D*A- aus einem freien, gelösten oxidierten

Donor und einem freien gelösten, reduzierten Akzeptor gebildet wird. Ein weiterer solcher äußerer Umstand kann - wie für die Elekron-Donor(en) und Elektron-Akzeptor(en) der chemisch induzierbar redoxaktiven Einheit - die Übertragung eines Elektrons auf den freien, gelösten Elektron-Donor durch ein Reduktionsmittel bzw. die Abgabe eines Elektrons durch den freien, gelösten Elektron-Akzeptor an ein Oxidationsmittel sein.

Die photoinduzierbar redoxaktive Einheit kann z. B. jedes beliebige photoinduzierbar redoxaktive Protein/Enzym oder jeder beliebige photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Im Ausdruck "photoinduzierbar redoxaktiver, verknüpfter, wenigstens Elekton-Donor-/Elektron-Akzeptor-Komplex" steht der Begriff bimolekularer "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn dieser Donor und dieser Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden sind. Durch Einstrahlung von Licht bestimmter oder beliebiger Wellenlänge gibt der/ein Elektron-Donor an einen der Elektron-Akzeptoren ein Elektron ab und es bildet sich, zumindest temporär, ein ladungsgetrennter Zustand D*A aus einem oxidierten Donor und einem reduzierten Akzeptor. Dieser Vorgang innerhalb der photoinduzierbar redoxaktiven Einheit wird als photoinduzierte Ladungstrennung bezeichnet. Bei entsprechend gewählten äußeren Umständen entfaltet die photoinduzierbar redoxaktive Einheit ihre Redoxaktivität, also ihre Eigenschaft, an einen geeignetes Oxidationsmittel Elektronen abzugeben oder von Reduktionsmittel einem aeeianeten. Elektronen aufzunehmen. ladungsgetrennten Zustand, da das Reduktionsmittel (bzw. Oxidationsmittel) nur auf den oxidierten Donor (bzw. vom reduzierten Akzeptor) der photoinduzierbar redoxaktiven Einheit Elektronen überträgt (bzw. aufnimmt), z. B. in Gegenwart eines Reduktionsmittels, das D*, jedoch nicht D, reduziert kann (bzw. in Gegenwart eines Oxidationsmittels das A-, jedoch nicht A, oxidieren kann). Insbesondere kann dieses Oxidations- bzw. Reduktionsmittel auch eine Elektrode sein, wobei die photoinduzierbar redoxaktive **Einheit** erst nach der photoinduzierten Ladungstrennung ein Elektron an eine Elektrode abgeben (bzw. von dieser aufnehmen), z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A., jedoch nicht A, oxidiert (bzw. D*, jedoch nicht D, reduziert) wird.

Die chemisch induzierbar redoxaktive Einheit kann z. B. jedes beliebige chemisch induzierbar redoxaktive Protein/Enzym oder jeder beliebige chemisch induzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elekton-Donor-/Elektron-Akzeptor-Komplex sein. Im Ausdruck "chemisch induzierbar redoxaktiver, verknüpfter, wenigstens bimolekularer Elekton-Donor-/Elektron-Akzeptor-Komplex" steht der

Begriff "wenigstens bimolekular" dafür, daß der Komplex aus wenigstens einem Elektron-Donor und wenigstens einem Elektron-Akzeptor aufgebaut ist, auch wenn dieser Donor und dieser Akzeptor direkt (oder indirekt über einen Spacer) kovalent verbunden sind. Bei entsprechend gewählten äußeren Umständen entfaltet die chemisch induzierbar redoxaktive Einheit ihre Redoxaktivität, also deren Eigenschaft unter bestimmten äußeren Umständen an ein geeignetes Oxidationsmittel Elektronen abzugeben (bzw. von einem geeigneten Reduktionsmittel Elektronen aufzunehmen), erst nach Reduktion (bzw. nach Oxidation) durch ein externes Reduktionsmittel (bzw. Oxidationsmittel). Erst nach Übertragung eines Elektrons von einem Reduktionsmittel auf den/einen Elektron-Donor "D" kann der dann reduzierte Donor "D" " ein Elektron auf den Akzeptor "A" übertragen und das Oxidationsmittel kann nur von diesem reduzierten Akzeptor "A-" der redoxaktiven Einheit Elektronen aufnehmen. Insbesondere kann das angesprochene Oxidationsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem A-, jedoch nicht A, oxidiert wird. Umgekehrt kann - bei abweichend gewählten äußeren Umständen - der Elektron-Akzeptor der chemisch induzierbar redoxaktive Einheit erst nach Übertragung eines Elektrons vom Akzeptor "A" auf ein externes Oxidationsmittel in seinem dann oxidierten Zustand "A* " ein Elektron vom Donor D aufnehmen und das Reduktionsmittel nur auf den oxidierten Donor "D" " der redoxaktiven Einheit Elektronen übertragen. Insbesondere kann das angesprochene Reduktionsmittel auch eine Elektrode sein, z. B. wenn die Elektrode auf ein Potential gesetzt wird, bei dem D*, jedoch nicht D, reduziert wird.

Mit dem Begriff "Oxidationsmittel" wird im Rahmen der vorliegenden Erfindung eine chemische Verbindung (chemische Substanz) bezeichnet, die durch Aufnahme von Elektronen aus einer anderen chemischen Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemischen Substanz, Elektron-Donor, Elektron-Akzeptor) oxidiert. Das Oxidationsmittel verhält sich analog zu einem Elektron-Akzeptor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht zur redoxaktiven Einheit gehörigen Elektron-Akzeptor verwendet. "Nicht unmittelbar" bedeutet in diesem Zusammenhang, daß das Oxidationsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das Oxidationsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäure-Oligomers, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- oder Purin-Basen von der kovalenten Anbindungstelle der redoxaktiven Einheit entfernt ist. Insbesondere kann die Elektrode das Oxidationsmittel darstellen.

Mit dem Begriff "Reduktionsmittel" wird im Rahmen der vorliegenden Erfindung eine chemische Verbindung (chemische Substanz) bezeichnet, die durch Abgabe von Elektronen an eine andere chemische Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) diese andere chemische Verbindung (chemische Substanz, Elektron-Donor, Elektron-Akzeptor) reduziert. Das Reduktionsmittel verhält sich analog zu einem Elektron-Donor, wird aber im Rahmen der vorliegenden Erfindung als Begriff für einen externen, nicht unmittelbar zur redoxaktiven Einheit gehörigen Elektron-Donor verwendet. "Nicht unmittelbar" bedeutet in diesem Zusammenhang, daß das Reduktionsmittel entweder eine freie redoxaktive Substanz ist, die nicht an das Nukleinsäure-Oligomer gebunden ist, aber mit diesem in Kontakt steht oder daß das Reduktionsmittel kovalent an das Nukleinsäure-Oligomer angebunden ist, jedoch an einer Stelle des Nukleinsäure-Oligomers, die mindestens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Nukleotide oder wenigstens zwei kovalent verbundene Pyrimidin- oder Purin-Basen von der kovalenten Anbindungstelle der redoxaktiven Einheit entfernt ist. Insbesondere kann die Elektrode das Reduktionsmittel darstellen.

Mit dem Begriff "freie redoxaktive Substanz" wird im Rahmen der vorliegenden Erfindung ein freies, nicht kovalent mit der redoxaktiven Einheit, dem Nukleinsäure-Oligomer oder der leitfähigen Oberfläche verbundenes, aber mit diesen, z. B. über die der modifizierten leitfähigen Oberfläche zugefügte Lösung, in Kontakt stehendes Oxidationsoder Reduktionsmittel bezeichnet, wobei die freie redoxaktive Substanz z. B. ein ungeladenes Molekül, eine beliebiges Salz oder ein redoxaktives Protein oder Enzym (Oxydoreductase) sein kann. Die freie redoxaktive Substanz ist dadurch gekennzeichnet, daß sie den oxidierten Donor (bzw. den reduzierten Akzeptor) der photoinduzierbar redoxaktive Einheit re-reduzieren (bzw. re-oxidieren) kann bzw. daß die freie, redoxaktive Substanz den Donor (bzw. den Akzeptor) der chemisch induzierbar redoxaktiven Einheit reduzieren (bzw. oxidieren) kann. Desweiteren ist die freie redoxaktive Substanz dadurch gekennzeichnet, daß sie bei einem Potential φ oxidierbar und reduzierbar ist, wobei φ der Bedingung 2,0 V $\geq \varphi \geq$ - 2,0 V genügt. Das Potential bezieht sich hierbei auf das freie redoxaktive Molekül in einem geeigneten Lösungsmittel, gemessen gegen Normalwasserstoffelektrode. Im Rahmen der vorliegenden Erfindung ist der Potentialbereich 1,7 V $\geq \varphi \geq$ - 1.7 V bevorzugt, wobei der Bereich 1,4 V $\geq \varphi \geq$ - 1,2 V besonders bevorzugt ist und der Bereich 0,9 V $\geq \varphi \geq$ - 0,7 V, in dem die redoxaktiven Substanzen der Anwendungsbeispiele oxidiert (bzw. reduziert) werden, ganz besonders bevorzugt ist.

Das modifizierte Nukleinsäure-Oligomer ist direkt oder indirekt (über einen Spacer) an eine leitfähige Oberfläche gebunden. Unter dem Begriff "leitfähige Oberfläche" wird jede elektrisch leitfähige Oberfläche beliebiger Dicke verstanden, insbesondere

metallische Oberflächen, Oberflächen aus Metallegierungen oder dotierte oder nicht dotierte Halbleiteroberflächen, wobei sämtliche Halbleiter als Reinsubstanzen oder als Gemische Verwendung finden können. Die leitfähige Oberfläche kann im Rahmen der vorliegenden Erfindung alleine oder auf einem beliebigen Trägermaterial, wie z. B. Glas, aufgebracht vorliegen. Im Rahmen der vorliegenden Erfindung wird der Begriff "Elektrode" alternativ zu "leitfähige Oberfläche" gebraucht.

Unter dem Begriff "modifizierte leitfähige Oberfläche" wird eine leitfähige Oberfläche verstanden, die durch Anbindung eines mit einer redoxaktiven Einheit modifizierten Nukleinsäure-Oligomers modifiziert ist.

Gemäß eines weiteren Aspekts betrifft die vorliegende Erfindung ein Verfahren, das die elektrochemische Detektion molekularer Strukturen, insbesondere die elektrochemische Detektion von DNA-/RNA-/PNA-Fragmenten in einer Probenlösung durch sequenzspezifische Nukleinsäure-Oligomer-Hybridisierung ermöglicht. Die Detektion der Hybridisierungsereignisse durch elektrische Signale ist eine einfache und kostengünstige Methode und ermöglicht in einer batteriebetriebenen Variante den Einsatz vor Ort.

Außerdem stellt die vorliegende Erfindung ein photoadressierbares Ausleseverfahren zur Detektion molekularer Strukturen zur Verfügung, unter anderem zur Detektion von Hybridisierungsereignissen auf einem Oligomer-Chip durch z. B. elektrische Signale. Erfindungsgemäß wird unter photoadressierbarem (Oligomer-Chip-) Ausleseverfahren ein Verfahren verstanden, bei dem die Detektion molekularer Strukturen auf ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe innerhalb des Gesamtsystems (des kompletten Oligomer-Chips) begrenzt wird, indem Licht bestimmter oder beliebiger Wellenlänge zur Induktion der Redoxaktivität der photoinduzierbar redoxaktiven Einheit räumlich auf diese Test-Site (-Gruppe) fokussiert (begrenzt) wird.

Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer

Voraussetzung für das erfindungsgemäße Verfahren ist die Bindung einer photoinduzierbar redoxaktiven Einheit bzw. einer chemisch induzierbar redoxaktiven Einheit an ein Nukleinsäure-Oligomer.

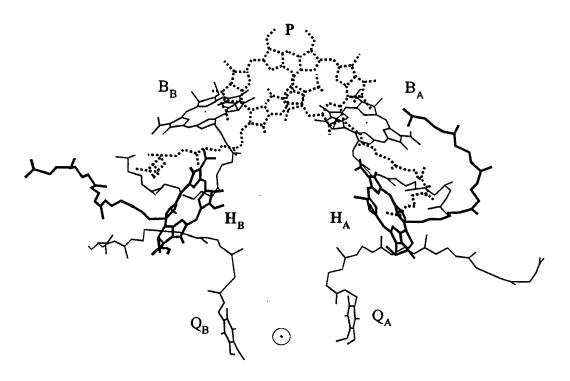
Als Beispiele einer photoinduzierbar redoxaktiven Einheit seien genannt:

(i) das photosynthetische bakterielle Reaktionszentrum (RC), wie z. B. das RC von Rhodobacter sphaeroides mit der schematischen Struktur 1, das RC anderer photosynthetischer Bakterien, wie z. B. das Reaktionszentrum von Rhodopseudomonas viridis oder von Rhodobacter capsulatus, oder ein Reaktionszentrum der Photosynthese betreibenden Pflanzen, wie z. B das Photosystem 1 oder das Photosystem 2, als Beispiele für ein photoinduzierbar redoxaktives Protein/Enzym.

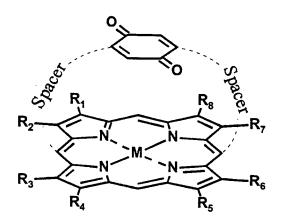
- (ii) Cyclophane, also verbrückte Porphyrin-Chinon-Systeme, der allgemeinen Struktur 2 als Beispiel für einen photoinduzierbar redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Flektron-Akzeptor-Komplex. Die beiden Spacerverbrückten, kovalenten Verbindungen ("- - Spacer - -" in Struktur 2) zwischen dem Elektron-Akzeptor (1,4-Benzochinon in der Struktur 2) und dem Elektron-Donor (Metallo-Porphyrin in der Struktur 2) können an beliebigen Stellen des Elektron-Donors und/oder Elektron-Akzeptors angebracht sein. Neben den in der Struktur ? gezeigten Elektron-Akzeptoren können auch Flavine der allgemeinen Formel ', Nicotinsäureamide der allgemeinen Formel 2 oder andere Chinone, z. B. solche der allgemeinen Formeln 3 - 8 oder organische bzw. anorganische Elektron-Akzeptoren und außerdem neben den (Metallo-) Porphyrinen der allgemeinen Formel 9 andere Elektron-Donoren, wie z. B. (Metallo-)Chlorophylle der allgemeinen Formel 10 oder (Metallo-) Bakteriochlorophylle der allgemeinen Formel 11 oder andere organische bzw. anorganische Elektron-Donoren verwendet werden. Daneben können auch einfach kovalent (Spacer-)verbrückte Elektron-Donor/Elektron-Akzeptor-Komplexe wie z. B. kovalente Verbindungen einer Substanz gemäß Formel 9 und einer der Substanzen gemäß einer der Formeln 1 - 8, kovalente Verbindungen einer Substanz gemäß Formel 10 und einer der Substanzen gemäß einer der Formeln 1-8 oder kovalente Verbindungen einer Substanz gemäß Formel 11 und einer der Substanzen gemäß einer der Formeln 1 - 8 als photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekulare Elektron-Donor/Elektron-Akzeptor-Komplexe verwendet werden.
- (iii) photoinduzierbar redoxaktive, verknüpfte, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplexe bei denen (einer) der Elektron-Donor(en) und/oder (einer) der Elektron-Akzeptor(en) ein Charge-Transfer-Komplex oder Übergangsmetall-Komplex ist. Beispiele für Übergangsmetall-Komplexe sind [Ru(bipy)₂(py)(im)]²⁺, beliebige andere [Ru(II)(L1)(L2)(L3)(L4)(L5)(L6)]-Komplexe, Cr(III)-, Fe(II)-, Os(II)-, oder Co(II)-Komplexe, wobei "bipy" für einen Bispyridyl-Liganden, "py" für einen Pyridyl-Liganden, "im" für einen Immidazol-Liganden und L1

bis L3 für einen beliebigen Liganden steht und auch mehr oder weniger als 6 Liganden an ein Übergangsmetall koordinieren können.

Beispiele einer chemisch induzierbar redoxaktiven Einheit sind der Cytochrom-bc Komplex oder der Cytochrom c₂ Komplex der Photosynthese betreibenden Bakterien (Komplex aus Proteinmatrix und vier eingebetteten Fe-Porphyrin Cofaktoren als Elektron-Donoren und/oder Elektron-Akzeptoren) als Beispiele für ein chemisch induzierbar redoxaktives Protein/Enzym oder, wie unter (ii) und (iii) aufgelistet, geeignet zusammengesetzte Cyclophane bzw. analoge Verbindungen als Beispiele für einen chemisch induzierbar redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex.



Struktur 1: Reaktionszentrum bestehend aus den Cofaktoren P (primärer Donor, ein Bakteriochlorophyll Dimer), B_A und B_B (Bakteriochlorophyll Monomere), H_A und H_B (Bakteriopheophytine), Q_A und Q_B (Ubichinon-50) und den Proteinuntereinheiten L, M, und H (nicht gezeigt), die die Cofaktoren einhüllen.



Struktur 2: ein Cyclophan; M = z. B. 2H, Mg, Zn, Cu, Ni, Pd, Co, Cd, Mn, Fe, Sn, Pt etc.; R_1 bis R_8 , oder Spacer sind unabhängig voneinander beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.

$$R_2$$
 R_3
 R_4
 R_5
 R_5

Formel 1

$$R_2$$
 R_3
 N
 R_4
 N
 R_5

Formel 2

$$R_2$$
 R_3
 R_1
 R_5
 R_4

Formel 3

$$R_1$$
 R_2
 R_3

Formel 4

Formel 5

$$R_2$$
 R_3
 R_4
 R_5
 R_6

Formel 6

$$R_2$$

$$R_3$$

$$R_4$$

$$R_5$$

Formel 7

$$R_2$$

$$R_3$$

$$R_4$$

$$R_5$$

$$R_8$$

$$R_7$$

$$R_6$$

Formel 8

 R_1 bis R_8 sind unabhängig voneinander H oder beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.

$$R_{12}$$
 R_{12}
 R_{12}
 R_{13}
 R_{14}
 R_{10}
 R

Formel 10

$$R_{12}$$
 R_{12}
 R_{13}
 R_{4}
 R_{4}
 R_{4}
 R_{10}
 R_{1

Formel 11

M=2H, Mg, Zn, Cu, Ni, Pd, Co, Cd, Mn, Fe, Sn, Pt etc.; R_1 bis R_{12} sind unabhängig voneinander H oder beliebige Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinyl-Substituenten.

Daneben zeichnet sich die redoxaktive Einheit erfindungsgemäß dadurch aus, daß besagte Einheit an ein ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenes Oxidationsmittel Elektronen abgibt bzw. von einen anderen ebenfalls kovalent an das Oligonukleotid angebundenen Reduktionsmittel Elektronen aufnimmt, wobei dieses Oxidations- oder Reduktionsmittel insbesondere eine elektrisch leitfähige Oberfläche (Elektrode) sein kann und die redoxaktive Einheit durch Anlegen einer äußeren Spannung an dieser Elektrode im elektrochemisch zugänglichen Potentialbereich der Elektrode elektrooxidiert/-reduziert werden kann.

Die redoxaktive Substanz zeichnet sich erfindungsgemäß dadurch aus, daß sie die photoinduzierbar redoxaktive Einheit, nach deren Elektron-Abgabe an ein anderes, von der redoxaktiven Substanz verschiedenes, kovalent an das Oligonukleotid angebundenes Oxidationsmittel re-reduzieren kann (bzw. nach deren Elektron-Aufnahme von einem anderen, von der redoxaktiven Substanz verschiedenen. kovalent an das Oligonukleotid angebundenen Reduktionsmittel re-oxidieren kann). bzw. daß die freie, redoxaktive Substanz den Donor (bzw. den Akzeptor) der chemisch induzierbar redoxaktiven Einheit reduzieren (bzw. oxidieren) kann. Erfindungsgemäß kann dazu jede redoxaktive Substanz verwendet werden, solange sie bei einem Potential ϕ , das der Bedingung 2,0 V $\geq \phi \geq$ - 2,0 V genügt, oxidierbar und reduzierbar ist und das Potential geeignet ist, besagte photoinduzierbar redoxaktive Einheit nach deren Elektron-Abgabe an ein anderes, ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenes Oxidationsmittel zu re-reduzieren (bzw. nach deren Aufnahme eines Elektrons von einen anderen, ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenen Reduktionsmittel zu re-oxidieren) oder besagte chemisch induzierbar redoxaktive Einheit zu reduzieren bzw. zu oxidieren. Das Potential bezieht sich hierbei auf die freie, unmodifizierte, redoxaktive Substanz in einem geeigneten Lösungsmittel, gemessen gegen Normalwasserstoffelektrode. Im Rahmen der vorliegenden Erfindung ist der Potentialbereich 1,7 V $\geq \phi \geq$ - 1.7 V bevorzugt, wobei der Bereich 1,4 V $\geq \phi \geq$ - 1,2 V besonders bevorzugt ist und der Bereich 0,9 V $\geq \phi \geq$ - 0,7 V, in dem die redoxaktiven Substanzen des Anwendungsbeispiels oxidiert (und rereduziert) werden, ganz besonders bevorzugt ist. Geeignet sind, neben den üblichen organischen und anorganischen redoxaktiven Molekülen wie z. B. Hexacyanoferraten, Ferrocenen, Cobaltocenen und Chinonen, vor allem die Ascorbinsäure (oder das Na⁺ Salz davon), [Ru(NH₃)₆]²⁺, Cytochrom c₂ (cyt c₂)²⁺, ein frei bewegliches eisenhaltiges Protein, das den oxidierten primären Donor P* in RC von Rhodobacter sphaeroides zu P reduziert und dabei selbst zu (cvt c2)3+ oxidiert wird.

In einer bevorzugten Ausführungsform der vorliegenden Erfindung ist der photo- oder chemisch induzierbar redoxaktive, verknüpfte, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex dergestalt in ein oder mehrere Makromoleküle eingebettet, daß das Makromolekül als elektrisch isolierende Einhüllende des redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplexes wirkt, indem er die direkte Elektrooxidation/-reduktion des redoxaktiven, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplexes an der Elektrode , z. B. bei direktem Kontakt zwischen Elektrode und redoxaktivem, verknüpften, wenigstens bimolekularen Elektron-Donor/Elektron-Akzeptor-Komplex, verhindert, die indirekte, über doppelsträngiges Nukleinsäure-Oligomer vermittelte Elektrooxidation/-reduktion Elektron-Donor/Elektron-Akzeptor-Komplexes aber erlaubt. Ein solchen Makromolekül kann z. B. ein maßgeschneidertes Cyclodextrin sein, das durch seine Form eines abgeschnittenen, innen hohlen Kegels ein Cyclophan oder ähnliche Elektron-Donor/Elektron-Akzeptor-Komplex ummantelt.

Erfindungsgemäß wird eine redoxaktive Einheit an ein Nukleinsäure-Oligomer kovalent durch die Reaktion des Nukleinsäure-Oligomers mit der redoxaktiven Einheit oder Teilen davon (siehe auch Abschnitt "Wege zur Ausführung der Erfindung") gebunden. Diese Bindung kann auf vier verschiedene Arten durchgeführt werden:

a) Als reaktive Gruppe zur Bindungsbildung am Nukleinsäure-Oligomer wird eine freie Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe des Oligonukleotid-Rückgrats, insbesondere eine Gruppe an einem der beiden Enden des Oligonukleotid-Rückgrats, verwendet. Die freien, endständigen Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen weisen eine erhöhte Reaktivität auf und gehen daher leicht typische Reaktionen wie z. B. Amidbildung mit (primären oder sekundären) Aminogruppen bzw. mit Säuregruppen, Esterbildung mit (primären, sekundären oder tertiären) Alkoholen bzw. mit Säuregruppen, Thioesterbildung mit (primären, sekundären oder tertiären) Thio-Alkoholen bzw. mit Säuregruppen oder die Kondensation von Amin und Aldehyd mit anschließender Reduktion der entstandenen CH=N Bindung zur CH2-NH Bindung ein. Die zur kovalenten Anbindung der redoxaktiven Einheit nötige Kopplungsgruppe (Säure-, Amin-, Alkohol-, Thioalkohol- oder Aldehydfunktion) ist entweder natürlicherweise an der redoxaktiven Einheit vorhanden oder wird durch chemische Modifikation der redoxaktiven Einheit erhalten. Die Anbindung der redoxaktiven Einheit kann komplett oder in Teilen der Einheit mit anschließender Vervollständigung der redoxaktiven Einheit erfolgen (siehe unten).

b) Das Nukleinsäure-Oligomer ist über einen kovalent angebundenen Molekülteil (Spacer) beliebiger Zusammensetzung und Kettenlänge (längste durchgehende Kette von aneinander gebundenen Atomen), insbesondere der Kettenlänge 1 bis 14, am Oligonukleotid-Rückgrat bzw. an einer Base mit einer reaktiven Gruppe modifiziert. Die Modifikation erfolgt bevorzugt an einem der Enden des Oligonukleotid-Rückgrats bzw. an einer terminalen Base. Als Spacer kann z.B. ein Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinylsubstituent verwendet werden. Mögliche einfache Reaktionen zur Ausbildung der kovalenten Bindung zwischen redoxaktiver Einheit und des so modifizierten Nukleinsäure-Oligomers sind wie unter a) beschrieben, die Amidbildung aus Säure- und Amino-Gruppe, die Esterbildung aus Säure- und Alkohol-Gruppe, die Thioesterbildung aus Säure- und Thio-Alkohol-Gruppe oder die Kondensation von Aldehyd und Amin mit anschließender Reduktion der entstandenen CH=N Bindung zur CH₂-NH Bindung. Die Anbindung der redoxaktiven Einheit kann komplett oder in Teilen der redoxaktiven Einheit mit anschließender Vervollständigung der Einheit erfolgen (siehe unten).

- c) Bei der Synthese des Nukleinsäure-Oligomers wird eine terminale Base durch die redoxaktive Einheit ersetzt. Diese Anbindung der redoxaktiven Einheit kann komplett oder in Teilen der Einheit mit anschließender Vervollständigung der redoxaktiven Einheit erfolgen (siehe unten).
- d) Bei der Verwendung eines verknüpften (wenigstens bimolekularen) Elekton-Donor-/Elektron-Akzeptor-Komplexes als redoxaktiver Einheit wird der Elektron-Akzeptor (oder -Donor) in einer ersten kovalenten Modifikation, wie unter b) oder c) in diesem Abschnitt beschrieben, an eine oder statt einer terminalen Base an das Nukleinsäure-Oligomer gebunden und anschließend in einer zweiten kovalenten Modifikation der Elektron-Donor (oder -Akzeptor), wie unter a) in diesem Abschnitt beschrieben, am selben Ende des Nukleinsäure-Oligomer-Rückgrats an eine reaktive Gruppe des Rückgrats oder an eine reaktive Gruppe des Akzeptors (bzw. des Donors) gebunden. Bei Verwendung eines kovalent verknüpften, tri- oder höhermolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes kann, statt des Elektron-Akzeptors (oder -Donors), auch ein beliebiger Teil des Elekton-Donor-/Elektron-Akzeptor-Komplexes in der ersten kovalenten Modifikation verwendet und in einer zweiten bzw. weiteren kovalenten Modifikation(en) komplettiert werden.

Erfindungsgemäß kann die Bindung der redoxaktiven Einheit an das Nukleinsäure-Oligomer ganz oder in Teilen vor oder nach der Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche erfolgen. So kann im Falle eines redoxaktiven Proteins/Enzyms aus Apoprotein und Cofaktor(en) statt der kompletten redoxaktiven

Einheit auch nur das Apoprotein, das Apoprotein und ein Teil der Cofaktoren oder ein oder mehrere Cofaktoren angebunden sein und die redoxaktive Einheit wird durch anschließende Rekonstitution mit den noch fehlenden Teilen komplettiert. Bei der Verwendung eines verknüpften (wenigstens bimolekularen) Elekton-Donor-/Elektron-Akzeptor-Komplexes als redoxaktiver Einheit wird der Elektron-Akzeptor (oder -Donor) in einer ersten kovalenten Modifikation, wie unter b) oder c) in diesem Abschnitt beschrieben, an oder statt einer terminalen Base an das Nukleinsäure-Oligomer gebunden und anschließend in einer zweiten kovalenten Modifikation der Elektron-Donor (oder -Akzeptor), wie unter a) in diesem Abschnitt beschrieben. am selben Ende des Nukleinsäure-Oligomer-Rückgrats an eine reaktive Gruppe des Rückgrats gebunden. Bei Verwendung eines kovalent verknüpften, tri- oder höhermolekularen Elekton-Donor-/Elektron-Akzeptor-Komplexes kann, statt des Elektron-Akzeptors (oder -Donors), auch ein beliebiger Teil des Elekton-Donor-/Elektron-Akzeptor-Komplexes in der ersten kovalenten Modifikation verwendet und in der zweiten kovalenten Modifikation komplettiert werden. Diese Modifikationen können vor oder nach der Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche erfolgen.

Bei mehreren verschiedenen Nukleinsäure-Oligomer-Kombinationen (Test-Sites) auf einer gemeinsamen Oberfläche ist es vorteilhaft, die (kovalente) Anbindung der redoxaktiven Einheit an die Nukleinsäure-Oligomere durch geeignete Wahl der reaktiven Gruppe an den freien Nukleinsäure-Oligomerenden der verschiedenen Test-Sites für die gesamte Oberfläche zu vereinheitlichen, wenn die redoxaktive Einheit nach Immobilisierung des Nukleinsäure-Oligomers an der Oberfläche angebunden werden soll.

Bei Verwendung von redoxaktiven Proteinen/Enzymen als redoxaktiver Einheit kann die kovalente Anbindung des Nukleinsäure-Oligomers an eine beliebige, natürlicherweise vorhandene oder durch Modifikation angebrachte, reaktive Gruppe des Proteins erfolgen oder - in dem Falle, daß das redoxaktive Protein/Enzym aus Apoprotein und Cofaktor(en) besteht - an eine beliebige, natürlicherweise vorhandene oder durch Modifikation angebrachte, reaktive Gruppe eines (beliebigen) Cofaktors. Im Rahmen der vorliegenden Erfindung ist die kovalente Anbindung an eine beliebige, natürlicherweise vorhandene oder durch Modifikation angebrachte, reaktive Gruppe eines (beliebigen) Cofaktors des Proteins bevorzugt. Ohne an mechanistische Details gebunden sein zu wollen, ist bei mehreren Cofaktoren derjenige besonders bevorzugt, der Elektronen an ein externes, ebenfalls kovalent an das Nukleinsäure-Oligomer angebundenen

Reduktionsmittel aufnehmen kann (siehe auch Abschnitt "Verfahren zur amperometrischen Detektion von Nukleinsäure-Oligomer-Hybriden").

Die leitfähige Oberfläche

Unter dem Begriff "leitfähige Oberfläche" wird erfindungsgemäß jeder Träger mit einer elektrisch leitfähigen Oberfläche beliebiger Dicke verstanden, insbesondere Oberflächen aus Platin, Palladium, Gold, Cadmium, Quecksilber, Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium und Mangan.

Daneben können auch beliebige dotierte oder nicht dotierte Halbleiteroberflächen beliebiger Dicke verwendet werden. Sämtliche Halbleiter können als Reinsubstanzen oder als Gemische Verwendung finden. Als nicht einschränkend gemeinte Beispiele seien an dieser Stelle Kohlenstoff, Silizium, Germanium, α-Zinn, Cu(I)- und Ag(I)-Halogenide beliebiger Kristallstruktur genannt. Geeignet sind ebenfalls sämtliche binären Verbindungen beliebiger Zusammensetzung und beliebiger Struktur aus den Elementen der Gruppen 14 und 16, den Elementen der Gruppen 13 und 15, sowie den Elementen der Gruppen 15 und 16. Daneben können ternäre Verbindungen beliebiger Zusammensetzung und beliebiger Struktur aus den Elementen der Gruppen 11, 13 und 16 oder den Elementen der Gruppen 12, 13 und 16 verwendet werden. Die Bezeichnungen der Gruppen des Periodensystems der Elemente beziehen sich auf die IUPAC-Empfehlung von 1985.

Bindung eines Nukleinsäure-Oligomers an die leitfähige Oberfläche

Erfindungsgemäß wird ein Nukleinsäure-Oligomer direkt oder über einen Linker/Spacer mit den Oberflächenatomen oder -molekülen einer leitfähigen Oberfläche der oben beschriebenen Art verknüpft. Diese Bindung kann auf drei verschiedene Arten durchgeführt werden:

a) Die Oberfläche wird so modifiziert, daß eine reaktive Molekül-Gruppe zugänglich ist. Dies kann durch direkte Derivatisierung der Oberflächenmoleküle, z. B. durch naßchemische oder elektrochemische Oxidation/Reduktion geschehen. So kann z. B. die Oberfläche von Graphitelektroden durch Oxidation naßchemisch mit Aldehydoder Carbonsäure-Gruppen versehen werden. Elektrochemisch besteht z. B. die Möglichkeit durch Reduktion in Gegenwart von Aryl-Diazoniumsalzen das entsprechende (funktionalisierte, also mit einer reaktiven Gruppe versehene) Aryl-

Radikal oder durch Oxidation in Gegenwart von R'CO₂H das (funktionalisierte) R'-Radikal auf der Graphit-Elektrodenoberfläche anzukoppeln. Ein Beispiel der direkten Modifikation von Halbleiteroberflächen ist die Derivatisierung von Siliziumoberflächen zu reaktiven Silanolen, d. h. Silizium-Träger mit Si-OR" Gruppen an der Oberfläche, wobei R" ebenso wie R' einen beliebigen, funktionalisierten, organischen Rest darstellt (z.B. Alkyl-, Alkenyl-, Alkinyl-, Heteroalkyl-, Heteroalkenyl- oder Heteroalkinylsubstituent). Alternativ kann die gesamte Oberfläche durch die kovalente Anbindung einer reaktiven Gruppe eines bifunktionalen Linkers modifiziert werden, so daß auf der Oberfläche eine monomolekulare Schicht beliebiger Moleküle entsteht, die, bevorzugt endständig, eine reaktive Gruppe enthalten. Unter dem Begriff "bifunktionaler Linker" wird jedes Molekül beliebiger Kettenlänge, insbesondere der Kettenlängen 2-14, mit zwei gleichen (homo-bifunktional) oder zwei verschiedenen (hetero-bifunktional) reaktiven Molekül-Gruppen verstanden.

Sollen mehrere verschiedene Test-Sites auf der Oberfläche durch Ausnutzen der Methodik der Photolithographie gebildet werden, so ist mindestens eine der reaktiven Gruppen des homo- oder hetereo-bifunktionalen Linkers eine photoinduzierbar reaktive Gruppe, d. h. eine erst durch Lichteinstrahlung bestimmter oder beliebiger Wellenlänge reaktiv werdende Gruppe. Dieser Linker wird so aufgebracht, daß die/eine photoaktivierbare reaktive Gruppe nach der kovalenten Anbindung des Linkers auf der Oberfläche zur Verfügung steht. An die so modifizierte Oberfläche werden die Nukleinsäure-Oligomere kovalent angebunden, wobei diese selbst über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, mit einer reaktiven Gruppe modifiziert sind, bevorzugt in der Nähe eines Endes des Nukleinsäure-Oligomers. Bei der reaktiven Gruppe des Oligonukleotids handelt es sich um Gruppen, die direkt (oder indirekt) mit der modifizierten Oberfläche unter Ausbildung einer kovalenten Bindung reagieren. Daneben kann an die Nukleinsäure-Oligomere in der Nähe ihres zweiten Endes eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die redoxaktive Einheit (komplett oder Bestandteile davon), alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Nukleinsäure-Oligomers angebunden sein.

b) Das Nukleinsäure-Oligomer, das auf die leitfähige Oberfläche aufgebracht werden soll, ist über einen kovalent angebundenen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, mit einer oder mehreren reaktiven Gruppen modifiziert, wobei sich die reaktive Gruppen bevorzugt in der

PCT/EP00/00084 WO 00/42217

Nähe eines Endes des Nukleinsäure-Oligomers befindet. Bei den reaktiven Gruppen handelt es sich um Gruppen, die direkt mit der unmodifizierten Oberfläche reagieren können. Beispiele hierfür sind: (i) Thiol- (HS-) oder Disulfid- (S-S-) derivatisierte Oligonukleotide der allgemeinen Formel HS-Spacer-oligo bzw. R-S-S-SpacerNukleinsäure-Oligomere der allgemeinen Formel (n x HS-Spacer)-oligo, (n x einer oligo-Spacer-S-S-Spacer-oligo, mit R-S-S-Spacer)-oligo oder einer Gold-Schwefelbindungvon Gold-Ausbildung unter Goldoberfläche Schwefelbindungen reagieren oder (ii) Amine, die sich durch Chemi- oder Physikesorption an Platin- oder Silizium-Oberflächen anlagern. Daneben kann an die OligonuklootidNukleinsäure-Oligomere in der Nähe ihres zweiten Endes (5'- oder 3'-Ende) die redexaktive Substanz oder eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die photinduzierbar redoxaktive Einheit (komplett oder Bestandteile davon) alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Oligonukleotids angebunden sein. Insbesondere Nukleinsäure-Oligomere die mit mehreren Spacer-verbrückten Thiol oder Disulfidbrücken modifiziert sind ((n x HS-Spacer)-oligo bzw. (n x R-S-S-Spacer)oligo) haben den Vorteil, daß solche Nukleinsäure-Oligomere unter einem bestimmten Anstellwinkel gegen die leitfähige Oberfläche (Winkel zwischen der Oberflächennormalen und der Helixachse eines doppelsträngigen helikalen Nukleinsäure-Oligomers bzw. zwischen der Oberflächennormalen und der Achse senkrecht zu den Basenpaaren eines doppelsträngigen nicht-helikalen Nukleinsäure-Oligomers) aufgebracht werden können, wenn die die Thiol- bzw. Disulfid-Funktionen an das Nukleinsäure-Oligomer anbindenden Spacer, von einem Ende der Nukleinsäure her betrachtet, eine zunehmende bzw. abnehmende Kettenlänge besitzen.

Sonden-Nukleinsäure-Oligomer werden am Gruppe Als reaktive Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen des Oligonukleotid-Rückgrats, insbesondere endständige Gruppen, verwendet. Die Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppen weisen eine erhöhte Reaktivität auf und gehen daher leicht typische Reaktionen wie z. B. mit (primären oder sekundären) Amino- bzw. Säuregruppen, Amidbildung sekundären oder tertiären) Alkoholen bzw. (primären, mit Esterbildung Säuregruppen, Thioesterbildung mit (primären, sekundären oder tertiären) Thio-Alkoholen bzw. Säuregruppen oder die Kondensation von Amin und Aldehyd mit anschließender Reduktion der entstandenen CH=N Bindung zur CH2-NH Bindung ein. Die nötige Kopplungs-Gruppe zur kovalenten Anbindung an die Phosphorsäure-,

Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe ist in diesem Fall ein Teil der Oberflächenderivatisierung mit einer (monomolekularen) Schicht beliebiger Moleküllänge, wie unter a) in diesem Abschnitt beschrieben, oder die Phosphorsäure-, Zucker-C-3-Hydroxy-, Carbonsäure- oder Amin-Gruppe kann direkt mit der unmodifizierten Oberfläche reagieren, wie unter b) in diesem Abschnitt beschrieben. Daneben kann an die Oligonukleotide in der Nähe ihres zweiten Endes eine weitere reaktive Gruppe gebunden sein, wobei diese reaktive Gruppe wiederum, wie oben beschrieben, direkt oder über einen Spacer beliebiger Zusammensetzung und Kettenlänge, insbesondere der Kettenlänge 1 - 14, angebunden ist. Desweiteren kann die redoxaktive Einheit (komplett oder Bestandteile davon), alternativ zu dieser weiteren reaktiven Gruppe, an diesem zweiten Ende des Nukleinsäure-Oligomers angebunden sein.

Die Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche kann vor oder nach der Anbindung der redoxaktive Einheit an das Nukleinsäure-Oligomer erfolgen. Im Falle eines redoxaktiven Proteins/Enzyms aus Apoprotein und Cofaktor(en) kann statt der kompletten redoxaktiven Einheit auch nur das Apoprotein, das Apoprotein mit einem Teil der Cofaktoren oder ein oder mehrere der Cofaktor angebunden sein und die redoxaktive Einheit wird durch anschließende Rekonstitution mit den noch fehlenden Teilen komplettiert. Bei der Verwendung eines verknüpften (wenigstens bimolekularen) Elekton-Donor-/Elektron-Akzeptor-Komplexes als redoxaktive Einheit kann der Elektron-Akzeptor (bzw. -Donor), wie unter b) oder c) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben, an eine oder statt einer terminalen Base an das Nukleinsäure-Oligomer gebunden sein und der Elektron-Donor (bzw. -Akzeptor) durch anschließende kovalente Anbindung an eine reaktive Gruppe des Elektron-Akzeptors (oder -Donors) angebunden werden oder, wie unter a) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben, durch anschließende Anbindung an eine terminale reaktive Gruppe des Nukleinsäure-Oligomer-Rückgrats am selben Ende (siehe auch den Abschnitt "Wege zur Ausführung der Erfindung"). Alternativ kann die Bindung des Nukleinsäure-Oligomers an die leitfähige Oberfläche vor oder nach Anbinden des mit einer reaktiven Gruppe versehenen Spacers zur Bindung der redoxaktiven Einheit erfolgen. Die Bindung des bereits modifizierten Nukleinsäure-Oligomers an die leitfähige Oberfläche, d. h. die Bindung an die Oberfläche nach der Anbindung der redoxaktiven Einheit an das Nukleinsäure-Oligomer bzw. nach der Anbindung von Teilen der redoxaktiven Einheit oder nach Anbinden des mit einer reaktiven Gruppe versehenen Spacers zur Bindung der redoxaktiven Einheit, erfolgt ebenfalls wie unter a) bis c) in diesem Abschnitt beschrieben.

Bei der Herstellung der Test-Sites muß bei der Anbindung der Einzelstrang-Nukleinsäure-Oligomere an die Oberfläche darauf geachtet werden, daß zwischen den einzelnen Nukleinsäure-Oligomeren ein genügend großer Abstand verbleibt, um zum einen den für eine Hybridisierung mit dem Target-Nukleinsäure-Oligomer nötigen Freiraum und zum anderen den für die Anbindung der redoxaktiven Einheit nötigen Freiraum zur Verfügung zu stellen. Dazu bieten sich insbesondere drei verschiedene Vorgehensweisen (und Kombinationen daraus) an:

- 1.) Herstellung einer modifizierten Oberfläche durch Anbindung eines hybridisierten Nukleinsäure-Oligomers, also eine Oberflächen-Derivatisierung mit hybridisiertem Sonden-Nukleinsäure-Oligomer statt mit Einzelstrang-Sonden-Oligonukleotid. Der zur Hybridisierung verwendete Nukleinsäure-Oligomer-Strang ist unmodifiziert (die Oberflächenanbindung wird durchgeführt wie unter a) c) in diesem Abschnitt beschrieben). Anschließend wird der hybridisierte Nukleinsäure-Oligomer-Doppelstrang thermisch dehybridisiert, wodurch eine mit Einzelstrang- Nukleinsäure-Oligomer modifizierte Oberfläche mit größerem Abstand zwischen den Sonden-Nukleinsäure-Oligomeren hergestellt wird.
- 2.) Herstellung einer modifizierten Oberfläche durch Anbindung eines Einzelstrangoder Doppelstrang-Nukleinsäure-Oligomers, wobei während der Oberflächen-Derivatisierung ein geeigneter monofunktionaler Linker zugesetzt wird, der neben dem Einzelstrang- oder Doppelstrang-Nukleinsäure-Oligomer ebenfalls an die Oberfläche gebunden wird (die Oberflächenanbindung wird durchgeführt wie unter a) - c) in diesem Abschnitt beschrieben). Erfindungsgemäß hat der monofunktionale Linker eine Kettenlänge, die der Kettenlänge des Spacers zwischen der Oberfläche und dem Nukleinsäure-Oligomer identisch ist oder um maximal vier Kettenatome abweicht. Bei der Verwendung von Doppelstrang-Nukleinsäure-Oligomer zur Oberflächen-Derivatisierung wird der Nukleinsäure-Oligomer-Doppelstrang nach der gemeinsamen Anbindung des Doppelstrang- Nukleinsäure-Oligomers und des Linkers an die Oberfläche thermisch dehybridisiert. Durch die gleichzeitige Anbindung eines Linkers an die Oberfläche wird der Abstand zwischen den ebenfalls an die Oberfläche gebundenen Einzel- oder Doppelstrang-Nukleinsäure-Oligomeren vergrößert. Im Falle der Verwendung von Doppelstrang-Nukleinsäure-Oligomer wird dieser Effekt durch die anschließende thermische Dehybridisierung noch verstärkt.
 - 3.) Herstellung einer modifizierten Oberfläche durch Anbindung eines Einzelstrangoder Doppelstrang-Oligonukleotids, an das die redoxaktive Einheit bereits angebunden ist, wobei die redoxaktive Einheit einen Durchmesser von größer als 30

Å aufweist. Bei der Verwendung von Doppelstrang-Oligonukleotid wird der Oligonukleotid-Doppelstrang nach der Anbindung des Doppelstrang-Oligonukleotids an die Oberfläche thermisch dehybridisiert.

Im Bezug auf die einzelnen Schritte zur "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" als auch zur "Bindung eines Oligonukleotids an die leitfähige Oberfläche" sei darauf verwiesen, daß im Abschnitt "Wege zur Ausführung der Erfindung" die verschiedenen Kombinationsmöglichkeiten der einzelnen Schritte, die zum selben Endergebnis führen, an einem Beispiel demonstriert sind (Figur 2).

Verfahren zur elektrochemischen Detektion von Nukleinsäure-Oligomer-Hybriden

Vorteilhafterweise werden gemäß dem Verfahren zur elektrochemischen Detektion Sonden-Nukleinsäure-Oligomere von Nukleinsäure-Oligomer-Hybriden mehrere nötigen Kombinationen des idealerweise alle Sequenz, unterschiedlicher Nukleinsäure-Oligomers, auf einem Oligomer (DNA) -Chip aufgebracht, um die Target-Nukleinsäure-Oligomers beliebigen Sequenz (fragmentierten) Target-DNA zu detektieren bzw. um Mutationen im Target aufzuspüren und sequenzspezifisch nachzuweisen. Dazu werden auf einer leitfähigen Oberfläche die Oberflächenatome oder -moleküle eines definierten Bereichs (einer Test-Site) mit DNA-/RNA-/PNA-Nukleinsäure-Oligomeren bekannter, aber beliebiger Sequenz, wie oben beschrieben, verknüpft. In einer allgemeinsten Ausführungsform kann aber der DNA-Chip auch mit einem einzigen Sonden-Oligonukleotid derivatisiert werden. Als Sonden- Nukleinsäure-Oligomere werden Nukleinsäure-Oligomere (z. B. DNA-, RNA- oder PNA-Fragmente) der Basenlänge 3 bis 50, bevorzugt der Länge 5 bis 30, besonders bevorzugt der Länge 8 bis 25 verwendet. Erfindungsgemäß wird oder ist an die Sonden-Nukleinsäure-Oligomere, wie nachfolgend beschrieben, eine redoxaktive Einheit gebunden.

Die Modifikation der Sonden-Nukleinsäure-Oligomere mit einer redoxaktiven Einheit kann komplett oder in Bestandteilen der redoxaktiven Einheit entweder vor oder nach der Bindung des Sonden-Oligonukleotids an die leitfähige Oberfläche erfolgen. Die verschiedenen Kombinationsmöglichkeiten der einzelnen Schritte (Reaktionssequenzen), sind mit Hilfe der Figur 2 am Beispiel einer über ein Sonden-Oligonukleotid an eine Elektrode gebundenen redoxaktiven Einheit im Abschnitt "Wege zur Ausführung der Erfindung" demonstriert.

Unabhängig von der jeweiligen Reaktionssequenz entsteht ein Oberflächen-Hybrid der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit, wobei "Einheit" repräsentativ für die photoinduzierbar redoxaktive Einheit bzw. chemisch induzierbar redoxaktive Einheit steht. Die Verbrückungen können natürlich auch ohne Spacer oder mit nur einem Spacer (Elek-ss-oligo-Spacer-Einheit bzw. Elek-Spacer-ss-oligo-Einheit) durchgeführt werden. Im Beispiel der Figur 2 ist die Einheit eine photoinduzierbar redoxaktive Einheit, nämlich das Reaktionszentrum (RC) der Photosynthese betreibenden Bakterien des Stammes Rhodobacter sphaeroides, ein photoinduzierbar redoxaktives Protein bestehend aus Apoprotein und Cofaktoren. Im Beispiel der Figur 2, 3 und 4 ist das RC über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten Q_A-Protein-Bindungstasche des RCs kovalent mit dem Nukleinsäure-Oligomer verbunden. Das RC bildet mit dem Cofaktor Ubichinon-50 in der QA-Bindungstasche einen 1:1 Komplex, wobei das Ubichinon-50 in der beschriebenen Weise kovalent an das Nukleinsäure-Oligomer gebunden ist. Im Beispiel der Figur 5 und 6 ist die Einheit ein photoinduzierbar redoxaktiver. verknüpfter, wenigstens bimolekularer Elektron-Donor-/Elektron-Akzeptor-Komplex, nämlich ein kovalent verknüpfter Zn-Bakteriochlorophyll-Chinon-Komplex, der über das Chinon, dem Elektron-Akzeptor-Molekül des Komplexes, kovalent (über einen Spacer) mit dem Nukleinsäure-Oligomer verbunden ist.

Die elektrochemische Kommunikation zwischen der (leitfähigen) Oberfläche und der über ein Einzelstrang-Oligonukleotid verbrückten redoxaktiven Einheit ("Einheit") in der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit ist schwach oder gar nicht vorhanden.

In einem nächsten Schritt werden die Test-Sites mit der zu untersuchenden Nukleinsäure-Oligomer-Lösung (Target) in Kontakt gebracht. Dabei kommt es nur in dem Fall zur Hybridisierung, in dem die Lösung Nukleinsäure-Oligomer-Stränge enthält, die zu den an die leitfähige Oberfläche gebundenen Sonden-Nukleinsäure-Oligomeren komplementär, oder zumindest in weiten Bereichen komplementär sind. Im Falle der Hybridisierung zwischen Sonden- und Target-Nukleinsäure-Oligomer kommt es zu einer verstärkten Leitfähigkeit zwischen der Oberfläche und der redoxaktiven Einheit, da diese nunmehr über das aus einem Doppelstrang bestehende Nukleinsäure-Oligomer verbrückt ist. Figur 3 zeigt dies schematisch am Beispiel der Elek-Spacer-ss-oligo-Spacer-UQ(RC). In Figur 4 ist die Sequenz der Elektron-Transfer-Schritte in Elek-Spacer-ds-oligo-Spacer-UQ(RC) im Detail gezeigt, während Figur 5 das Beispiel Elek-Spacer-ss-oligo-Spacer-Q-ZnBChl schematisch zeigt und Figur 6 die Sequenz der Elektron-Transfer-Schritte in Elek-Spacer-ds-oligo-Spacer-Q-ZnBChl im Detail darstellt.

Aufgrund der Hybridisierung von Sonden-Nukleinsäure-Oligomer und dem dazu komplementären Nukleinsäure-Oligomer-Strang (Target) verändert sich die elektrische Kommunikation zwischen der (leitfähigen) Oberfläche und der photoinduzierbar redoxaktiven Einheit. Somit kann ein sequenzspezifisches Hybridisierungsereignis durch elektrochemische Verfahren wie z. B. Cyclovoltametrie, Amperometrie oder Leitfähigkeitsmessungen detektiert werden.

Bei der Cyclovoltametrie wird das Potential einer stationären Arbeitselektrode zeitabhängig linear verändert. Ausgehend von einem Potential bei dem keine Elektrooxidation oder reduktion stattfindet, wird das Potential solange verändert bis die redoxaktive Substanz oxidiert oder reduziert wird (also Strom fließt). Nach Durchlaufen des Oxidations- bzw. Reduktionsvorgangs, der in der strom/Spannungskurve einen zunächst ansteigenden Strom, dann einen Maximalstrom (Peak) und schließlich einen allmählich abfallenden Strom erzeugt, wird die Richtung des Potentialvorschubs umgekehrt. Im Rücklauf wird dann das Verhalten der Produkte der Elektrooxidation oder -reduktion aufgezeichnet.

Eine alternative elektrische Detektionsmethode, die Amperometrie, wird dadurch ermöglicht, daß die redoxaktive Einheit durch Anlegen eines geeigneten, konstant gehaltenen Elektrodenpotentials zwar elektrooxidiert (elektroreduziert) werden kann, die Rereduktion (Reoxidation) der redoxaktiven Einheit in den ursprünglichen Zustand aber nicht wie in der Cyclovoltametrie durch Änderung des Elektrodenpotentials erfolgt, sondern durch ein der Targetlösung zugesetztes geeignetes Reduktionsmittel (Oxidationsmittel), der "redoxaktiven Substanz", wodurch der Stromkreis des Gesamtsystems geschlossen wird. Solange solches Reduktionsmittel (Oxidationsmittel) vorhanden ist bzw. solange das verbrauchte Reduktionsmittel (Oxidationsmittel) an der Gegenelektrode rereduziert (reoxidiert) wird, fließt Strom, der amperometrisch detektiert werden kann und der proportional zur Zahl der Hybridisierungsereignisse ist.

Dieses Prinzip der amperometrischen Detektion soll stellvertretend für eine photoinduzierbar redoxaktive Einheit bzw. für eine redoxaktive Einheit am Beispiel der Glucoseoxidase näher erläutert werden. Die Glucoseoxidase ist ein aus Apoprotein und <u>einem</u> Cofaktor (Flavin-Adenin-Dinukleotid) bestehendes redoxaktives Enzym. Das mit einem Ende kovalent an die Elektrode angebundene Sonden-Oligonukleotid kann am anderen, noch freien Ende mit der vollständigen enzymatischen Einheit der Glucoseoxidase funktionalisiert werden, indem z. B. der Flavin-Adenin-Dinukleotid (FAD)-Cofaktor des Enzyms kovalent an das Sonden-Oligonukleotid angebunden wird und anschließend mit dem Glucoseoxidase-Apoprotein (GOx) rekonstituiert wird. Das entstandene Oberflächen-Hybrid der

allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-FAD(GOx) weist zwischen Elektrode und FAD keine oder nur geringe Leitfähigkeit auf. Im Falle der Hybridisierung mit dem zu "ss-oligo" komplementären Target-Oligonukleotid wird die Leitfähigkeit deutlich erhöht wird. Bei Zusatz des Substrats Glucose zur Target-Oligonukleotid-Lösung wird das FAD der Gukoseoxidase (FAD(GOx)) zu FADH2 der Glucoseoxidase (FADH2(GOx)) reduziert, wobei Glucose zur Gluconsäure oxidiert wird. Liegt nun an der Elektrode ein geeignetes äußeres Potential an, so daß über das hybridisierte Oligonukleotid Elektronen von FADH2(GOx) an die Elektrode abgegeben werden und somit FADH2(GOx) zu FAD(GOx) reoxidiert wird (aber weder Glucose noch Gluconsäure bei diesem Potential elektrooxidiert oder -reduziert werden kann), fließt im System Elek-Spacer-ds-oligo-Spacer-FAD(GOx) solange Strom wie FAD(GOx) durch freie Glucose reduziert wird, d. h. bis die gesamte Glucose verbraucht ist bzw. für den Fall, daß an der Gegenelektrode ein Potential anliegt, bei dem Gluconsäure zu Glucose reduziert werden kann, solange wie Gluconsäure an der Gegenelektrode reduziert wird. Dieser Strom kann proportional Zahl der amperometrisch detektiert werden und ist zur Hybridisierungsereignisse.

Die für diese Erfindung relevanten photoinduzierbar redoxaktiven Einheiten bzw. chemisch induzierbar redoxaktiven Einheiten besitzen statt eines Elektron-Donors bzw. Elektron-Akzeptors jedoch mindestens einen Elektron-Donor und mindestens einen Elektron-Akzeptor.

Im Falle einer chemisch induzierbar redoxaktiven Einheit ist im Sinne der vorliegenden Erfindung mindestens ein Ladungstransferschritt zwischen Elektron-Donor(en) und Elektron-Akzeptor(en) zwischengeschaltet. Die freie redoxaktive Substanz, die D reduziert (bzw. A oxidiert) und somit einen Elektrontransfer von Dzu A unter Bildung von A (bzw. den Elektrontransfer von Dzu A unter Bildung von D) initiiert, ermöglicht es, die Elektrode auf ein Potential zu setzten, bei dem A (bzw. D) oxidiert (bzw. reduziert) werden kann. Dies hat den Vorteil, daß die Elektrode ein Potential besitzt, bei dem die direkte Reaktion der freien, redoxaktiven Substanz mit der Elektrode deutlich unterdrückt werden kann und hauptsächlich Elektronenübertragungen zwischen redoxaktiver Einheit und Elektrode detektiert werden.

Handelt es sich bei der redoxaktiven Einheit um eine photoinduzierbar redoxaktive Einheit, so wird die Redoxaktivität der Einheit erst durch Licht bestimmter oder beliebiger Wellenlänge ausgelöst. Erfindungsgemäß wird diese Eigenschaft dadurch ausgenutzt, daß die elektrochemische Detektion erst durch Einstrahlen von Licht auf

das Oberflächenhybrid der allgemeinen Struktur Elek-Spacer-ds-oligo-Spacer-Einheit (Oberflächenhybrid mit hybridisiertem Target) ausgelöst wird und maximal solange aufrechterhalten wird wie die Lichteinstrahlung andauert. Insbesondere bei der amperometrischen Detektion fließt somit bei Verwendung einer photoinduzierbar unter bestimmten äußeren Umständen, redoxaktiven Einheit, (längeranhaltend) Strom, wenn Licht auf das Oberfächenhybrid eingestrahlt wird. Solche äußere Umstände sind z. B. die Gegenwart eines geeigneten Reduktionsmittels (bzw. Oxidationsmittels), um einen durch Photoinduktion gebildeten, oxidierten Donor D* (bzw. reduzierten Akzeptor A-) der photoinduzierbar redoxaktiven Einheit zu reduzieren (bzw. zu reduzieren) und das Anliegen eines Potentials an der Elektrode, bei dem zwar ein durch Photoinduktion gebildeter reduzierter Akzeptor A- (bzw. oxidierter Donor D+) der photoinduzierbar redoxaktiven Einheit, nicht jedoch der nicht reduzierte Akzeptor A (bzw. der nicht oxidierte Donor D) oxidiert (bzw. reduziert) werden kann. Im Abschnitt "Wege zur Ausführung der Erfindung" wird dies anhand verschiedener Beispiele von Elek-Spacer-ss-oligo-Spacer-Einheit mit photoinduzierbar redoxaktiver Einheit näher erläutert. Somit kann die Detektion bei Verwendung einer photoinduzier as redoxaktiven Einheit auf eine bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe des Oligomer-Chips räumlich beschränkt werden, indem das Licht auf dieses Test-Site oder auf diese Test-Site-Gruppe begrenzt wird. Erfindungsgemäß können also verschiedene Test-Sites (Nukleinsäure-Oligomer-Kombinationen) eines Oligomer-Chips auf eine gemeinsame, durchgängige, elektrisch leitende Oberfläche aufgebracht werden. Ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe kann einfach durch Anlegen eines geeigneten äußeren Potentials an die (gesamte) Oberfläche bei Lichteinstrahlung auf genau dieses Test-Site oder diese Test-Site Gruppe adressiert und amperometrisch detektiert werden. Die verschiedenen Test-Sites müssen also nicht auf einzelnen, elektrisch voneinander isolierten und zum Anlegen eines Potentials und Auslesen des Stroms einzeln ansteuerbaren (Mikro-)Elektroden Verwendung der kann Darüberhinaus werden. aufgebracht Oberflächenhybriden der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit mit photoinduzierbar redoxaktiver Einheit und amperometrischer Detektion der sequenzspezifischen einzelnen der Detektion zur Ausleseprozess Hybridisierungsereignisse auf dem Oligomer-Chip dadurch optimiert werden, daß die Test-Sites durch entsprechende Fokusierung des Lichts erst grobgerastert ausgelesen werden und dann in den Rastern mit Hybridisierungsereignissen des Auflösungsvermögen sukzessive erhöht wird, also z. B. bei einem Oktamer-Chip mit 65.536 Test-Sites zuerst in z. B. 64 Gruppen von je 1024 Test-Sites ausgelesen wird, dann die Test-Site-Gruppen, die anhand der amperometrischen Messungen Hybridisierungsereignisse aufweisen, z. B. in 32 Gruppen von je 32 Test-Sites

durchgetestet werden und anschließend in den erneut Hybridisierungsereignisse aufweisenden Test-Site-Gruppen die Test-Sites einzeln ausgetestet werden. Die einzelnen Hybridisierungsereignisse können dadurch mit geringem experimentellen Aufwand schnell bestimmten Sonden-Oligomeren zugeordnet werden.

Kurze Beschreibung der Zeichnungen

Die Erfindung soll nachfolgend anhand von Ausführungsbeispielen im Zusammenhang mit den Zeichnungen näher erläutert werden. Es zeigen

- Fig. 1 Schematische Darstellung der Oligonukleotid-Sequenzierung durch Hybridisierung auf einem Chip;
- Herstellung des Fig. 2 Verschiedene Reaktionssequenzen zur Elek-Spacer-ss-oligo-Spacer-UQ(RC). Die Oberflächenhybrids photoinduzierbar redoxaktive Einheit in diesem Oberflächenhybrid ist das Reaktionszentrum (RC) der Photosynthese betreibenden Bakterien Rhodobacter sphaeroides. Dieses photoinduzierbar redoxaktive Protein besteht aus Apoprotein und Cofaktoren. Das RC ist über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten QA-Protein-Bindungstasche kovalent über einen Spacer mit dem Oligonukleotid verbunden;
- Fig. 3 Schematische Darstellung der photoinduzierten amperometrischen Meßmethode am Beispiel des Oberflächen-Hybrids Elek-Spacer-ssoligo-Spacer-UQ(RC) aus Figur 2 (hv: Einstrahlung von Licht, P: primärer Donor des RC, UQ: Ubichinon-50 Elektron Akzeptor in der Q_A-Protein-Bindungstasche des RC, Red/Ox: reduzierte bzw. oxidierte Form der freien, der Targetlösung zugesetzten redoxaktiven Substanz, z. B. cyt c₂²⁺, Natriumascorbat oder Fe(CN)₈²⁺, die die oxidierte Form P⁺ in den ursprünglich neutralen Zustand P rereduzieren können, E_{Ox}: Potential der Elektrode, bei dem UQ⁻ durch Elektronabgabe an die Elektode zu UQ oxidiert wird, "hv an": Beginn der Lichteinstrahlung, "hv aus": Ende der Lichteinstrahlung);

Fig. 4 Detaillierte schematische Darstellung des Oberflächenhybrids Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) der Figur 3 mit Gold Obertlächenmaterial, Mercaptoethanol als Spacer (-S-CH₂CH₂- Spacer) zwischen Elektrode und Oligonukleotid und -CH2-CH=CH-CO-NH-CH2-CH₂-NH- als Spacer zwischen dem Elektron-Akzeptor UQ und Oligonukleotid sowie die Darstellung der Sequenz der photoinduzierten Elektron-Transfer-Schritte. Das Apoprotein des RCs ist nur als Hülle (durchgezogene Linie) angedeutet (vgl. Struktur 1). Das 12 Bp Sonden-Oligonukleotid der exemplarischen Sequenz 5'-TAGTCGGAAGCA-3' ist, als Ausschnitt, im hybridisierten Zustand gezeigt;

- Fig. 5 Schematische Darstellung der photoinduzierten amperometrischen Meßmethode am Beispiel des Oberflächen-Hybrids Elek-Spacer-ssoligo-Spacer-Q-ZnBChl (hv: Einstrahlung von Licht, ZnBChl: das Elektron-Donor-Molekül Zn-Bakteriochlorophyll, Q: das Elektron-Akzeptor-Molekül Chinon, z. B. modifiziertes Anthrachinon oder PQQ, Red/Ox: reduzierte bzw. oxidierte Form der freien, der Targetlösung zugesetzten redoxaktiven Substanz, z. B. Fe(CN)₆²⁺, das die oxidierte Form des Elektron-Donors ZnBChl⁺ in den ursprünglich neutralen Zustand ZnBChl rereduzieren kann, E_{Ox}: Potential der Elektrode, bei dem Q⁻ durch Elektronabgabe an die Elektrode zu Q oxidiert wird, "hv an": Beginn der Lichteinstrahlung, "hv aus": Ende der Lichteinstrahlung);
- Fig. 6 Detaillierte schematische Darstellung des Oberflächenhybrids Au-S(CH₂)₂-ds-oligo-Spacer-Q-ZnBChl der Figur 5 mit Gold als Oberflächenmaterial, Mercaptoethanol als Spacer (-S-CH₂CH₂- Spacer) zwischen Elektrode und Oligonukleotid und -CH₂-CH=CH-CO-NH-CH₂-CH₂-NH- als Spacer zwischen dem Elektron-Akzeptor PQQ und Oligonukleotid sowie die Darstellung der Sequenz der photoinduzierten Elektron-Transfer-Schritte. Das 12 Bp Sonden-Oligonukleotid der exemplarischen Sequenz 5'-TAGTCGGAAGCA-3' ist, als Ausschnitt, im hybridisierten Zustand gezeigt.

Wege zur Ausführung der Erfindung

Eine Bildungseinheit einer exemplarischen Test-Site mit hybridisiertem Target, Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) der allgemeinen Struktur Elek-Spacer-ds-oligo-

Spacer-Einheit ist in Figur 4 dargestellt. Unter Bildungseinheit wird im Rahmen der vorliegenden Erfindung die kleinste sich wiederholende Einheit einer Test-Site verstanden. In dem Beispiel der Figur 4 ist die Oberfläche eine Gold-Elektrode. Die Verbindung zwischen Gold-Elektrode und Sonden-Oligonukleotid wurde mit dem Linker (HO-(CH₂)₂-S)₂ aufgebaut, der mit der endständigen Phosphatgruppe am 3' Ende zu P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert wurde und nach homolytischer Spaltung der S-S Bindung an der Gold-Oberfläche je eine Au-S Bindung bewirkte, womit 2-Hydroxymercaptoethanol und Mercaptoethanol-verbrücktes Oligonukleotid auf der Oberfläche koadsorbiert wurde. Die photoinduzierbar redoxaktive Einheit im Beispiel der Figur 4 ist das Reaktionszentrum (RC) der Photosynthese betreibenden Bakterien Rhodobacter sphaeroides, ein photoinduzierbar redoxaktives Protein bestehend aus Apoprotein und Cofaktoren. Im Anwendungsbeispiel ist das RC über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten Q_A-Bindungstasche des RCs kovalent mit dem Oligonukleotid verbunden, wobei zuerst freies UQ mit einer reaktiven Carbonsäuregruppe versehen wurde (siehe Beispiel 1), dann freies UQ über diese Carbonsäure-Gruppe kovalent an das Sonden-Oligonukleotid angebunden wurde (Amidbildung unter Wassembspaltung mit der terminalen Aminofunktion des an die C-5-Position des 5'-Thymins angebundenen -CH=CH-CO-NH-CH2-CH2-NH2 Linkers) und schließlich das restliche RC (Apoprotein mit allen Cofaktoren außer UQ) an UQ rekonstituiert wurde.

Wie weiter oben bereits erwähnt, kann die Modifikation der Sonden-Oligonukleotide mit der kompletten oder mit einem Bestandteil der redoxaktiven Einheit entweder vor oder nach der Bindung des Sonden-Oligonukleotids an die leitfähige Oberfläche erfolgen. Die verschiedenen Kombinationsmöglichkeiten der einzelnen Schritte, die prinzipiell zur selben Bildungseinheit einer Test-Site führen, sollen im folgenden mit Hilfe der Figur 2 am Beispiel des Oberflächenhybrids Au-S(CH₂)₂-ss-oligo-Spacer-UQ(RC) bzw. in seiner allgemeineren Form als Elek-Spacer-ss-oligo-Spacer-UQ(RC) dargestellt werden.

Das Reaktionszentrum kann durch einfache Manipulation von den beiden Ubichinon-Cofaktoren in der Q_A - bzw. Q_B -Bindungstasche befreit werden (Gunner, M.R., Robertson, D.E., Dutton, P.L.,1986, Journal of Physical Chemistry, Vol. 90, S. 3783-3795), so daß man Ubichinon getrennt vom restlichen RC (Apoprotein einschließlich aller Cofaktoren außer Ubichinon in der Q_A - bzw. Q_B -Bindungstasche) erhält. Das Sonden-Oligonukleotid ist in der Nähe der beiden Enden jeweils über einen (beliebigen) Spacer mit (gleichen oder verschiedenen) reaktiven Gruppe versehen. In einer Reaktionssequenz "1" kann das so modifizierte Sonden-Oligonukleotid in Gegenwart eines monofunktionalen Linkers (entsprechend den Punkten a) - c) und

2.) im Abschnitt "Bindung eines Oligonukleotids an die leitfähige Oberfläche") gemeinsam mit dem monofunktionalen Linker kovalent an die Elektrode angebunden werden, wobei darauf geachtet wird, daß genügend monofunktionaler Linker geeigneter Kettenlänge zugesetzt wird, um zwischen den einzelnen Sonden-Oligonukleotiden genügend Freiraum für eine Hybridisierung mit dem Target-Oligonukleotid und für die Anbindung der redoxaktiven Einheit zur Verfügung zu stellen. Danach wird an die freie, spacerverbrückte, reaktive Gruppe des Sonden-Oligonukleotids UQ, das vorher mit einer passenden reaktiven Kopplungsgruppe versehen wurde, angebunden. Die Anbindung erfolgt wie unter a) bzw. b) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben. Im letzten Schritt dieser Reaktionssequenz "1" wird dann das restliche RC (Apoprotein mit allen Cofaktoren außer UQ) an UQ rekonstituiert. In einer Variante dazu (Reaktionssequenz "2") kann das (mit Spacer und reaktiven Gruppen) modifizierte Sonden-Oligonukleotid zuerst ohne freien, monofunktionalen Linker (Spacer) kovalent an die Elektrode gebunden werden, wobei es zu einer flachen Anlagerung des Oligonukleotids kommt. Danach wird der freie, monofunktionale Linker (Spacer) kovalent an die Elektrode gebunden. Eine weitere Möglichkeit (Reaktionssequenz "3") besteht darin, das (mit Spacer und reaktiven Gruppen) modifizierte Sonden-Oligonukleotid zuerst mit UQ zu modifizieren, dann in Gegenwart von freiem, monofunktionalen Linker (Spacer) kovalent an die Elektrode anzubinden und anschließend mit dem restlichen RC zu rekonstituieren. Schließlich kann in einer Reaktionssequenz "4" das (mit Spacer und reaktiven Gruppen) modifizierte Sonden-Oligonukleotid zuerst mit UQ modifiziert werden, um es dann mit dem restlichen RC zu rekonstituieren und anschließend kovalent an die Elektrode zu binden. Falls, wie im Fall des RCs, die redoxaktive Einheit einen wesentlich größeren Durchmesser aufweist als das hybridisierte ds-Oligonukleotid (größer als 30 Å), kann auf die kovalente Anbindung eines geeigneten freien, monofunktionalen Linkers (Spacers) an die Elektrode verzichtet werden, anderenfalls geschieht die Anbindung der Struktur -Spacer-ss-oligo-Spacer-UQ(RC) an die Elektrode in Gegenwart eines geeigneten, freien monofunktionalen Linkers.

Im Beispiel der Figur 2 ist das RC über seinen Cofaktor Ubichinon-50 (UQ) in der sogenannten Q_A-Protein-Bindungstasche des RCs kovalent mit dem Oligonukieotid verbunden. Alternativ kann statt des UQ-Cofaktors in der Q_A-Bindungstasche auch ein anderer Cofaktor des RCs oder das Apoprotein kovalent an das Sonden-Oligonukleotid angebunden werden, es können beliebige Kombinationen der Reaktionssequenzen "1", "2", "3" oder "4" in Figur 2 angewandt werden, solange sie zum gleichen Endprodukt führen (vgl. Figur 2) und es kann in beliebigen Reaktionsschritten statt des Einzelstrang-Sonden-Oligonukleotids das mit

komplementären, unmodifizierten (Target-)Oligonukleotid hybridisierte Sonden-Oligonukleotid verwendet werden. Das Sonden-Oligonukleotid kann auch direkt, also nicht über einen Spacer verbrückt, sowohl an die Elektrode als auch an die redoxaktive Einheit angebunden werden, wie unter c) im Abschnitt "Bindung eines Nukleinsäure-Oligomers an die leitfähige Oberfläche" bzw. a) im Abschnitt "Bindung einer redoxaktiven Einheit an ein Nukleinsäure-Oligomer" beschrieben.

Die elektrische Kommunikation zwischen der leitfähigen Oberfläche und der über ein Einzelstrang-Oligonukleotid verbrückten redoxaktiven Einheit in der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit ist schwach oder gar nicht vorhanden. Durch Behandlung der Test-Site(s) mit einer zu untersuchenden Oligonukleotid-Lösung, kommt es, im Falle der Hybridisierung zwischen Sonde und Target, zu einer verstärkten Leitfähigkeit zwischen der Oberfläche und der über ein Doppelstrang-Oligonukleotid verbrückten redoxaktiven Einheit. Für die Bildungseinheit der exemplarische Test-Site Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) (mit 12-Bp Sonden-Oligonukleotiden) ist dies schematisch in Figur 3 anhand amperometrischer Messungen gezeigt.

Durch Lichteinstrahlung geeigneter Wellenlänge auf das RC wird der Cofaktor P, der sogenannte primäre Donor, elektronisch angeregt und es kommt innerhalb der Cofaktoren des RCs zur photoinduzierten Ladungstrennung, wobei ein Elektron vom angeregten primären Donor P* auf das UQ in der QA-Bindungstasche übertragen wird. Liegt an der Elektrode ein geeignetes Potential an, um vom reduzierten Ubichinon (UQ⁻) ein Elektron auf die Elektrode zu übertragen, kommt es im Falle des nicht mit Target-Oligonukleotid hybridisierten Sonden-Oligonukleotids trotzdem zu keinem Stromfluß, da die Leitfähigkeit des ss-Oligonukleotids in Au-S(CH₂)₂-ss-oligo-Spacer-UQ(RC) sehr gering oder überhaupt nicht vorhanden ist. Im hybridisierten Zustand (Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC)) jedoch ist die Leitfähigkeit hoch, ein Elektron kann von UQ- zur Elektrode übertragen werden (unter Bildung von UQ) und bei Anwesenheit einer geeigneten redoxaktiven Substanz, die P⁺ zu P zu reduziert, wird der Stromkreis geschlossen und weitere Lichtabsorption durch das RC startet den Zyklus erneut. Dies äußert sich amperometrisch in einem deutlichen Stromfluß zwischen Elektrode und photoinduzierbar redoxaktiver Einheit (Figur 3). Damit ist es möglich, die sequenzspezifische Hybridisierung des Targets mit den Sonden-Oligonukleotiden durch Amperometrie lichtinduziert zu detektieren. Die einzelnen Elektron Transfer Schritte, die im Oberflächenhybrid Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) durch Lichteinstrahlung und bei Anwesenheit einer geeigneten redoxaktiven Substanz zur Reduktion von P* zu P ausgelöst werden, sind in Figur 4 dargestellt. Natürlich kann das Oberflächenhybrid Au-S(CH₂)₂-ds-oligo-Spacer-UQ(RC) unter

geeigneten äußeren Umständen und bei geeigneter Anbindung (z. B. Anbindung des RCs an das Sonden-Oligonukleotid in der Nähe des primären Donors), auch umgekehrt geschaltet werden, so daß nach Lichteinstrahlung P⁺ von der Elektrode reduziert und Q⁻ von einem geeigneten Oxidationsmittel oxidiert wird.

Eine weiteres Test-Site, Au-S(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl, der allgemeinen Struktur Elek-Spacer-ss-oligo-Spacer-Einheit ist in Figur 5 dargestellt. Durch Lichteinstrahlung geeigneter Wellenlänge auf ZnBChl wird ZnBChl elektronisch angeregt und es kommt zur photoinduzierten Ladungstrennung, wobei ein Elektron vom angeregten ZnBChl* auf das Chinon Q übertragen wird. Liegt an der Elektrode ein geeignetes Potential an, um vom so reduzierten Chinon (Q-) ein Elektron auf die Elektrode zu übertragen, kommt es im Falle des nicht mit Target-Oligonukleotid hybridisierten Sonden-Oligonukleotids trotzdem zu keinem Stromfluß, da die Leitfähigkeit des ss-Oligonukleotids in Au-S(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl sehr gering oder überhaupt nicht vorhanden ist. Im hybridisierten Zustand Au-S(CH₂)₂-dsoligo-Spacer-Q-ZnBChl jedoch ist die Leitfähigkeit hoch, ein Elektron kann von Qzur Elektrode übertragen werden (unter Bildung von Q) und bei Anwesenheit einer geeigneten redoxaktiven Substanz, die ZnBChl zu ZnBChl reduziert, wird der Stromkreis geschlossen und weitere Lichtabsorption durch ZnBChl startet den Zyklus erneut. Dies äußert sich amperometrisch in einem deutlichen Stromfluß zwischen Elektrode und photoinduzierbar redoxaktiver Einheit (Figur 5). Damit ist es möglich, die sequenzspezifische Hybridisierung des Targets mit den Sonden-Oligonukleotiden Amperometrie lichtinduziert zu detektieren. Natürlich Oberflächenhybrid Au-S(CH₂)₂-ds-oligo-Spacer-Q-ZnBChl unter geeigneten äußeren Umständen und geeigneter Anbindung (z. B. Au-S(CH₂)₂-ds-oligo-Spacer-ZnBChl-Q) auch umgekehrt geschaltet werden, so daß nach Lichteinstrahlung ZnBChl* von der Elektrode reduziert und Q- von einem geeigneten Oxidationsmittel oxidiert wird.

Da die Redoxaktivität der photoinduzierbar redoxaktiven Einheit - auch bei passendem Elektrodenpotential - erst durch Lichteinstrahlung geeigneter Wellenlänge ausgelöst und maximal solange aufrechterhalten wird, wie die Lichteinstrahlung andauert, kann dies erfindungsgemäß dadurch ausgenutzt werden, daß ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppe eines Oligomer-Chips räumlich aufgelöst wird, indem das Licht auf dieses Test-Site oder auf diese Test-Site-Gruppe begrenzt wird. Dies birgt den erfindungsgemäßen Vorteil, daß die verschiedenen Test-Sites (Nukleinsäure-Oligomer-Kombinationen) eines Oligomer-Chips auf eine gemeinsame, durchgängige, elektrisch leitende Oberfläche aufgebracht werden können und ein bestimmtes Test-Site oder eine bestimmte Test-Site-Gruppen einfach durch Anlegen eines geeigneten äußeren Potentials an die

PCT/EP00/00084 WO 00/42217

(gesamte) Oberfläche bei Lichteinstrahlung nur auf genau dieses Test-Site oder diese Test-Site Gruppe adressiert und amperometrisch detektiert werden kann. Die verschiedenen Test-Sites müssen also nicht auf einzelnen, elektrisch voneinander isolierten und zum Anlegen eines Potentials und Auslesen des Stroms einzeln ansteuerbaren (Mikro-) Elektroden aufgebracht werden.

Daneben können fehlerhafte Basenpaarungen (Basenpaar Mismatches) durch eine geänderte cyclovoltammetrische Charakteristik erkannt werden. Ein Mismatch äußert sich in einem größeren Potentialabstand zwischen den Strommaxima der Elektroreduktion und der Elektroreoxidation (Umkehrung der Elektroreduktion bei umgekehrter Potentialvorschubrichtung) bzw. der Elektrooxidation und Elektrorereduktion in einem cyclovoltammetrisch reversiblen Elektronen-Transfer zwischen der elektrisch leitenden Oberfläche und der photoinduzierbar redoxaktiven Einheit. Dieser Umstand wirkt sich vor allem in der amperometrischen Detektion günstig aus, da dort der Strom bei einem Potential getestet werden kann, bei dem zwar das perfekt hybridisierende Oligonukleotid-Target signifikant Strom liefert, nicht aber das fehlerhaft gepaarte Oligonukleotid-Target.

Beispiel 1: Modifikation des Ubichinon-50 mit einer Spacer-verbrückten reaktiven Carbonsäure-Gruppe. Die 2-Methoxy-Gruppe des Ubichinon-50 (UQ-50) wird durch Etherspaltung mit HBr, einer Standardmethode, zur 2-Hydroxygruppe modifiziert (alternativ kann 2-OH-UQ-50 nach dem Verfahren von Moore, H. W. and Folkers, K. Journal of the American Chemical Society, 1966, 88, 564-570 oder von Daves, G. et al., Journal of the American Chemical Society, 1968, 90, 5587-5593 hergestellt werden). Anschließend wird 2-OH-UQ-50 in einem Standardverfahren mit einer äquimolaren Menge an CI-CH2-CH2-CO2H zum 2-(CH2-CH2-CO2H)-UQ-50 umgesetzt 5-OH-6-alkyl-1.4können Alternativ aufgereinigt. chromatographisch Benzochinon-Analoga des UQ-50 (Darstellung gemäß Catlin et al., Journal of the American Chemical Society, 1968, 90, 3572-3574) in einem Standardverfahren mit einer äquimolaren Menge an CI-CH2-CH2-CO2H zu 5-(CH2-CH2-CO2H)-UQ-50-Analoga modifiziert werden.

Beispiel 2: Herstellung der Oligonukleotid-Elektrode Au-S(CH₂)₂-ss-oligo-SpacerUQ(RC). Die Herstellung von Au-S(CH₂)₂-ss-oligo-Spacer-UQ(RC) gliedert sich in 4 Teilabschnitte, nämlich der Darstellung der leitfähigen Oberfläche, der Derivatisierung der Oberfläche mit dem Sonden-Oligonukleotid in Gegenwart eines geeigneten monofunktionalen Linkers (Inkubationsschritt), der kovalenten Anbindung des modifizierten Ubichinon-50 (Redoxschritt) und der Rekonstitution des restlichen RCs (Rekonstitutionsschritt).

Das Trägermaterial für die kovalente Anbindung der Doppelstrang-Oligonukleotide bildet ein ca. 100 nm dünner Gold-Film auf Mica (Muskovit Plättchen). Dazu wurde in einer elektrischen Entladungskammer frisch gespaltenes Mica mit einem Argonlonenplasma gereinigt und durch elektrische Entladung Gold (99.99%) in einer Schichtdicke von ca. 100nm aufgebracht. Anschließend wurde der Gold-Film mit 30 % H_2O_2 , / 70 % H_2SO_4 von Oberflächenverunreinigungen befreit (Oxidation organischer Ablagerungen) und für ca. 20 Minuten in Ethanol getaucht, um an der Oberfläche adsorbierten Sauerstoff zu verdrängen. Nach Abspülen der Oberfläche mit bidestilliertem Wasser wird auf die horizontal gelagerte Oberfläche eine vorher bereitete 1×10^{-4} molare Lösung des (modifizierten) Doppelstrang-Oligonukleotids aufgetragen, so daß die komplette Gold-Oberfläche benetzt wird (Inkubationsschritt, siehe auch unten).

Zur Inkubation wurde ein doppelt modifiziertes 12 Bp Einzelstrang-Oligonukleotid der Sequenz 5'-TAGTCGGAAGCA-3' verwendet, das an der Phosphatgruppe des 3' Endes mit (HO-(CH₂)₂-S)₂ zum P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert ist. Am 5'-Ende ist die endständige Base Thymin des Oligonukleotids am C-5 Kohlenstoff mit -CH=CH-CO-NH-CH₂-CH₂-NH₂ modifiziert. Zu einer 2x10⁻⁴ molaren Lösung dieses Oligonukleotids in HEPES-Puffer (0,1 molar in Wasser, pH 7.5 mit 0.7 molarem Zusatz von TEATFB, siehe Abkürzungen) wurde ca. 10⁻⁴ bis 10⁻¹ molar 2-Hydroxy-mercaptoethanol gegeben (oder ein anderer Thiol- oder Disulfid-Linker geeigneter Kettenlänge) und die Gold-Oberfläche eines Test-Sites komplett benetzt und 2-24h inkubiert. Während dieser Reaktionszeit wird der Disulfidspacer P-O-(CH₂)₂-S-S-(CH₂)₂-OH des Oligonukleotids homolytisch gespalten. Dabei bildet der Spacer mit Au-Atomen der Oberfläche eine kovalente Au-S Bindung aus, wodurch es zu einer 1:1 Koadsorption des ss-Oligonukleotids und des abgespaltenen 2-Hydroxy-mercaptoethanols kommt. Das in der Inkubationslösung gleichzeitig anwesende, freie 2-Hydroxy-mercaptoethanol einer Au-S Bindung koadsorbiert wird ebenfalls durch Ausbildung (Inkubationsschritt).

Die so mit einer Monolayer aus ss-Oligonukleotid und 2-Hydroxy-mercaptoethanol modifizierte Goldelektrode wurde mit bidestilliertem Wasser gewaschen und anschließend mit einer Lösung von 3x10⁻³ molarem Chinon 2-(CH₂-CH₂-CO₂H)-UQ-50, 10⁻² molarem EDC und 10⁻² molarem sulfo-NHS in HEPES-Puffer (0,1 molar (in Wasser, pH = 7.5), benetzt. Nach einer Reaktionszeit von ca. 1 - 4 h bilden der - CH=CH-CO-NH-CH₂-CH₂-NH₂ Spacer und das 2-(CH₂-CH₂-CO₂H)-UQ-50 eine kovalente Bindung aus (Amidbildung zwischen der Aminogruppe des Spacers und der C-2-Säurefunktion des 2-(CH₂-CH₂-CO₂H)-UQ-50, Redoxschritt).

Anschließend wurde die so modifizierte Goldelektrode mit bidestilliertem Wasser gewaschen und mit einer Lösung von ca. 5x10⁻⁵ molarem Ubichinon-50-freien RCs in 10 mM Tris, pH = 8, mit 0.7 molarem Zusatz von TEATFB bei ca. 4 °C für ca. 12 h inkubiert, um das restliche RC an das Oligonukleotid-gebundene UQ-50 zu rekonstituieren (Rekonstitutionsschritt).

Alternativ zur kovalenten Anbindung von 2-(CH₂-CH₂-CO₂H)-UQ-50 an das Sonden-Oligonukleotid kann, unter gleichen Bedingungen, auch ein 5-(CH₂-CH₂-CO₂H)-UQ-50-Analogon (Beispiel 1) oder ein anderes, mit einer reaktiven Carbonsäure versehenes Chinon der Formel 1 - 8 verwendet werden, da auch an diese Ubichinon-50-freies RC rekonstituiert werden kann.

Beispiel 3: Herstellung der Oligonukleotid-Elektrode Au-S(CH2)2-ss-oligo-Spacer-Q-ZnBChl. Die Herstellung von Au-S(CH₂)₂-ss-oligo-Spacer-Q-ZnBChl gliedert sich in 5 der leitfähigen Oberfläche, Darstellung nämlich der Teilabschnitte, Derivatisierung der Oberfläche mit dem (mit Komplementärstrang hybridisierten) Sonden-Oligonukleotid in Gegenwart eines geeigneten monofunktionalen Linkers Elektron-Akzeptors des Anbindung kovalenten (Inkubationsschritt), der (Akzeptorschritt), der kovalenten Anbindung des Elektron-Donors (Donorschritt) und Doppelstrang-Oligonukleotids Dehybridisierung des der thermischen (Dehybridisierungsschritt).

Das Trägermaterial für die kovalente Anbindung der Doppelstrang-Oligonukleotide, ein ca. 100 nm dünner Gold-Film auf Mica (Muskovit Plättchen), wurde wie in Beispiel 1 beschrieben, hergestellt.

Zur Inkubation wurde ein doppelt modifiziertes 12 Bp Einzelstrang-Oligonukleotid der Sequenz 5'-TAGTCGGAAGCA-3' verwendet, das an der Phosphatgruppe des 3' Endes mit (HO-(CH₂)₂-S)₂ zum P-O-(CH₂)₂-S-S-(CH₂)₂-OH verestert ist. Am 5'-Ende ist die endständige Base Thymin des Oligonukleotids am C-5 Kohlenstoff mit -CH=CH-CO-NH-CH₂-CH₂-NH₂ modifiziert. Eine 2x10⁻⁴ molare Lösung dieses Oligonukleotids im Hybridisierungspuffer (10 mM Tris, 1 mM EDTA, pH 7.5 mit 0.7 molarem Zusatz von TEATFB, siehe Abkürzungen) wurde mit einer 2x10⁻⁴ molaren Lösung des Hybridisierungspuffer bei Strangs im komplementären (unmodifizierten) Raumtemperatur für ca. 2h hybridisiert (Hybridisierungsschritt). Nach der Hybridisierung wurde der nun 1x10⁻⁴ molaren Doppelstrang-Oligonukleotid-Lösung ca. 10⁻⁴ bis 10⁻¹ molar 2-Hydroxy-mercaptoethanol (oder ein anderer Thiol- oder Disulfid-Linkers geeigneter Kettenlänge) zugesetzt, die Gold-Oberfläche eines Test-Sites komplett benetzt und 2-24h inkubiert. Während dieser Reaktionszeit wird der Disulfidspacer P-O-(CH₂)₂-S-S-(CH₂)₂-OH des Oligonukleotids homolytisch gespalten. Dabei bildet der Spacer mit Au-Atomen der Oberfläche eine kovalente Au-S Bindung aus, wodurch es zu einer 1:1 Koadsorption des ds-Oligonukleotids und des abgespaltenen 2-Hydroxy-mercaptoethanols kommt. Das in der Inkubationslösung gleichzeitig anwesende, freie 2-Hydroxy-mercaptoethanol wird ebenfalls durch Ausbildung einer Au-S Bindung koadsorbiert (Inkubationsschritt).

Die so mit einer Monolayer aus ds-Oligonukleotid und 2-Hydroxy-mercaptoethanol modifizierte Goldelektrode wurde mit bidestilliertem Wasser gewaschen und anschließend mit einer Lösung von 3x10⁻³ molarem Chinon PQQ, 10⁻² molarem EDC und 10⁻² molarem sulfo-NHS in HEPES Puffer benetzt. Nach einer Reaktionszeit von ca. 1 - 4 h bilden der -CH=CH-CO-NH-CH₂-CH₂-NH₂ Spacer und das PQQ eine

kovalente Bindung (Amidbildung zwischen der Aminogruppe des Spacers und der C-7-Carbonsäurefunktion des PQQ, Akzeptorschritt).

Anschließend wurde die so modifizierte Goldelektrode mit bidestilliertem Wasser gewaschen und mit einer wässerigen Lösung aus 3x10-3 molarem Donor ZnBChl (freie Säure), 1,5x10⁻¹ molarem EDC, 2,5x10⁻³ molarem Hydrazin-Monohydrat (NH₂-NH₂xH₂O) und 1x10⁻¹ molarem Imidazol benetzt. Nach einer Reaktionszeit von ca. 16 h bei 23 °C bindet die C-1-Carbonsäurefunktion des an das Oligonukleotid gebundenen PQQ über Hydrazin an die freie Carbonsäure-Gruppe des ZnBChl (Amidbildung zwischen den Aminogruppen des Hydrazins und der C-1-Carbonsäure-Gruppe des PQQ bzw. der freien Carbonsäure-Gruppe des ZnBChl, Donorschritt). Anschließend wurden die Doppelstränge bei Temperaturen von T > 40 °C thermisch bidestilliertem Wasser mit dehybridisiert und emeut (Dehybridisierungsschritt). Das ZnBChl (freie Säure) wird aus Zn-BChl (Darstellung gemäß Hartwich et al., Journal of the American Chemical Society, 1998, 120, 3684-3693) durch Inkubation mit Trifluoressigsäure hergestellt.

PCT/EP00/00084

WO 00/42217

(Probe-Oligonukleotid-gebundenes) Q

Alternativ kann z. B. ZnBChl (freie Säure) über Esterbildung nach Standardverfahren auch an die 3-OH-Gruppe des 5'-terminalen Zuckers des Sonden-Oligonukleotids gebunden werden oder der vorher kovalent verbundene Elektron-Donor/Elektron-Akzeptor-Komplex wird, wie im Donorschritt beschrieben, über eine freie Carbonsäure-Gruppe z. B. des Donors an das Sonden-Oligonukleotid angebunden. Statt PQQ kann unter den gleichen Reaktionsbedingungen auch Anthrachinon-2,6-Disulfonsäure Dinatriumsalz im Akzeptorschritt verwendet werden. Bei Verwendung von PNA-Oligonukleotid mit z. B. -NH-(CH₂)₂-N(COCH₂-Base)-CH₂CO- als Oligonukleotid-Baustein besteht eine alternative Anbindungsmöglichkeit der ZnBChl-PQQ-Einheit an das Nukleinsäure-Oligomer (PNA-Oligonukleotid) entsprechend d) im Abschnitt "Bindung einer photoinduzierbar redoxaktiven Einheit an ein Nukleinsäure-Oligomer". Dabei wird während der PNA-Oligonukleotid-Synthese statt der N-terminale Base in der Standard-PNA-Synthese-Reaktion PQQ über seinen Pyrrol-Stickstoff angebunden. Anschließend wird Zn-BChl, ähnlich wie im Donorschritt beschrieben, durch Inkubation des mit PQQ modifizierten PNA-

Oligonukleotids mit 3x10⁻³ molarem ZnBChl (freie Säure), 1,5x10⁻¹ molarem EDC 10⁻² und 2x10⁻¹ molarem sulfo-NHS in HEPES Puffer an das Amino-Ende des Peptid-Rückgrats gebunden (Amidbildung zwischen der Aminogruppe des Rückgrats und der Carbonsäure-Gruppe des Zn-BChl (freie Säure)).

Patentansprüche

- Durch kovalente Anbindung einer redoxaktiven Einheit modifiziertes Nukleinsäure-Oligomer, dadurch gekennzeichnet, daß die redoxaktive Einheit ein oder mehrere Elektron-Donor-Moleküle und ein oder mehrere Elektron-Akzeptor-Moleküle enthält.
- 2.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 1, dadurch gekennzeichnet, daß die redoxaktive Einheit wenigstens einen redoxaktiven, verknüpften, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplex enthält, wobei wenigstens zwei der Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-wolekül(e) der redoxaktiven Einheit durch eine oder mehrere Bindungen miteinander verbunden sind.
- 3.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 2, dadurch gekennzeichnet, daß wenigstens zwei der Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-Molekül(e) der redoxaktiven Einheit durch eine oder mehrere kovalente Bindungen miteinander verbunden sind.
- 4.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 1, dadurch gekennzeichnet, daß die redoxaktive Einheit wenigstens einen redoxaktiven, verknüpften, wenigstens bimolekularen Elekton-Donor-/Elektron-Akzeptor-Komplex enthält, wobei wenigstens zwei der Elektron-Donor-Molekül(e) und/oder Elektron-Akzeptor-Molekül(e) durch einen oder mehrere verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent verbunden sind.
- 5.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 4, wobei die verzweigten oder unverzweigten Molekülteile eine Kettenlänge von 1 20 Atomen, ir besondere 1 14 Atomen, aufweisen.
- 6.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die ein oder mehrere Elektron-Donor-Moleküle und ein oder mehrere Elektron-Akzeptor-Moleküle enthaltende redoxaktive Einheit zusätzlich ein oder mehrere Makromoleküle umfaßt.
- 7.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei die redoxaktive Einheit das native oder modifizierte

Reaktionszentrum von Photosynthese betreibenden Organismen ist, insbesondere das native oder modifizierte Reaktionszentrum von Photosynthese betreibenden Bakterien.

- 8.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß eines oder mehrere der Elektron-Donor-und/oder Elektron-Akzeptor-Molekül(e) Farbstoffe sind, insbesondere Flavine, (Metallo-)Porphyrine, (Metallo-)Chlorophylle oder (Metallo-)Bakteriochlorophylle bzw. Derivate davon.
- 9.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß eines oder mehrere der Elektron-Donor-und/oder Elektron-Akzeptor-Molekül(e) Nikotinsäureamide oder Chinone sind, insbesondere Pyrrolo-Chinolin-Chinone (PQQ), 1,2-Benzochinone, 1,4-Benzochinone, 1,2-Naphtochinone, 1,4-Naphtochinone oder 9,10-Anthrachinone bzw. Derivate davon.
- 10.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß eines oder mehrere der Elektron-Donorund/oder Elektron-Akzeptor-Molekül(e) Charge-Transfer-Komplexe sind.
- 11.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 10, wobei der Charge-Transfer-Komplex ein Übergangsmetall-Komplex ist, insbesondere ein Ru(II)-, ein Cr(III)-, ein Fe(II)-, ein Os(II)- oder ein Co(II)-Komplex.
- 12.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei das modifizierte Nukleinsäure-Oligomer sequenzspezifisch Einzelstrang-DNA, RNA und/oder PNA binden kann.
- 13.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 12, wobei das modifizierte Nukleinsäure-Oligomer ein Desoxyribonukleinsäure-, Ribonukleinsäure-, ein Peptidnukleinsäure-Oligomer oder ein Nukleinsäure-Oligomer mit strukturell analogem Rückgrat ist.
- 14.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, wobei die redoxaktive Einheit kovalent alternativ an eine der Phosphorsäure-, Carbonsäure- oder Amin-Gruppen oder an einen Zucker, insbesondere an eine Zucker-Hydroxy-Gruppe, des Nukleinsäure-Oligomer-Rückgrats gebunden ist.

15.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 - 13, wobei die redoxaktive Einheit alternativ kovalent an eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.

- 16.) Modifiziertes Nukleinsäure-Oligomer nach Anspruch 15, dadurch gekennzeichnet, daß die reaktive Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe der Base kovalent über einen verzweigten oder unverzweigten Molekülteil beliebiger Zusammensetzung und Kettenlänge an die Base gebunden ist, wobei die kürzeste durchgehende Verbindung zwischen der Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe und der Base ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen, insbesondere von 1 14 Atomen, ist.
- 17.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 14 16, wobei die redoxaktive Einheit an ein Ende des Nukleinsäure-Oligomer-Rückgrats bzw. an eine endständige, modifizierte Base angebunden ist.
- 18.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die redoxaktive Einheit eine photoinduzierbar redoxaktive Einheit ist.
- 19.) Modifiziertes Nukleinsäure-Oligomer nach einem der Ansprüche 1 17, dadurch gekennzeichnet, daß die redoxaktive Einheit eine chemisch induzierbar redoxaktive Einheit ist.
- 20.) Modifiziertes Nukleinsäure-Oligomer nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß mehrere redoxaktive Einheiten an das Nukleinsäure-Oligomer angebunden sind.
- 21.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers wie in einem der vorhergehenden Ansprüche definiert, wobei eine redoxaktive Einheit kovalent an ein Nukleinsäure-Oligomer angebunden wird.
- 22.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 21, wobei die redoxaktive Einheit durch kovalente Anbindung eines oder mehrerer Elektron-Donor-Molekül(e) an ein Nukleinsäure-Oligomer angebunden wird.

23.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 21, wobei die redoxaktive Einheit durch kovalente Anbindung eines oder mehrerer Elektron-Akzeptor-Molekül(e) an ein Nukleinsäure-Oligomer angebunden wird.

- 24.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 21, wobei die redoxaktive Einheit durch kovalente Anbindung eines oder mehrerer Makromoleküle bzw. durch kovalente Anbindung eines oder mehrerer Proteine an ein Nukleinsäure-Oligomer angebunden wird.
- 25.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach den Ansprüchen 22 24, wobei die redoxaktive Einheit durch Zugabe von einem oder mehreren Elektron-Akzeptor-Molekül(en), einem oder mehreren Elektron-Donor-Molekül(en), einem oder mehreren Makromolekülen und/oder einem oder mehreren Proteinen vervollständigt wird.
- 26.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 21 25, wobei das Nukleinsäure-Oligomer alternativ durch eine oder mehrere Amidbildungen mit Amin- oder mit Säure-Gruppen der redoxaktiven Einheit, durch eine oder mehrere Esterbildungen mit Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit, durch Thioesterbildung mit Thio-Alkohol- oder mit Säure-Gruppen der redoxaktiven Einheit bzw. durch Kondensation einer oder mehrerer Amin-Gruppen des Nukleinsäure-Oligomers mit Aldehyd-Gruppen der redoxaktiven Einheit und anschließender Reduktion der entstandenen Kohlenstoff-Stickstoff-Doppelbindung an die redoxaktive Einheit gebunden wird.
- 27.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach einem der Ansprüche 21 26, wobei an die redoxaktive Einheit kovalent eine oder mehrere verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge angebunden ist und die verzweigten oder unverzweigten Molekülteile alternativ eine reaktive Amin-, Hydroxy-, Thiol-, Säure- oder Aldehyd-Gruppe zur kovalenten Anbindung an ein Nukleinsäure-Oligomer besitzen.
- 28.) Verfahren zur Herstellung eines modifizierten Nukleinsäure-Oligomers nach Anspruch 27, wobei die kürzeste durchgehende Verbindung zwischen dem Nukleinsäure-Oligomer und der redoxaktiven Einheit ein verzweigtes oder



unverzweigtes Molekülteil mit einer Kettenlänge von 1 - 20 Atomen, insbesondere von 1 - 14 Atomen, ist.

- 29.) Modifizierte leitfähige Oberfläche, dadurch gekennzeichnet, daß eine oder mehrere Arten von modifizierten Nukleinsäure-Oligomeren gemäß einem der Ansprüche 1 bis 20 an eine leitfähige Oberfläche angebunden sind.
- 30.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einem Metall oder einer Metallegierung besteht, insbesondere einem Metall ausgewählt aus der Gruppe Platin, Palladium, Gold, Cadmium, Quecksilber, Nickel, Zink, Kohlenstoff, Silber, Kupfer, Eisen, Blei, Aluminium, Mangan und deren Mischungen.
- 31.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einem Halbleiter besteht, insbesondere einem Halbleiter ausgewählt aus der Gruppe Kohlenstoff, Silizium, Germanium und -Zinn.
- 32.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einer binären Verbindung der Elemente der Gruppen 14 und 16, einer binären Verbindung der Elemente der Gruppen 13 und 15, einer binären Verbindung der Elemente der Gruppen 15 und 16, oder einer binären Verbindung der Elemente der Gruppen 11 und 17 besteht, insbesondere aus einem Cu(I)-Halogenid oder einem Ag(I)-Halogenid.
- 33.) Modifizierte leitfähige Oberfläche nach Anspruch 29, wobei die Oberfläche aus einer ternären Verbindung der Elemente der Gruppen 11, 13 und 16 oder einer ternären Verbindung Elemente der Gruppen 12, 13 und 16 besteht.
- 34.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 29 33, wobei die Anbindung der modifizierten Nukleinsäure-Oligomere an die leitfähige Oberfläche kovalent oder durch Chemi- bzw. Physisorption erfolgt.
- 35.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 34, wobei alternativ eine der Phosphorsäure-, Carbonsäure-, Amin- oder eine Zucker-Gruppe, insbesondere eine Zucker-Hydroxy-Gruppe, des Nukleinsäure-Oligomer-Rückgrats kovalent oder durch Chemi- bzw. Physisorption an die leitfähige Oberfläche angebunden ist.



- 36.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 34, dadurch gekennzeichnet, daß alternativ eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers kovalent oder durch Chemi- bzw. Physisorption an die leitfähige Oberfläche angebunden ist.
- 37.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 35 oder 36, wobei das modifizierte Nukleinsäure-Oligomer über eine Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats bzw. über eine Gruppe einer endständigen, modifizierten Base an die leitfähige Oberfläche gebunden ist.
- 38.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 29 37, wobei an die leitfähige Oberfläche verzweigte oder unverzweigte Molekülteile beliebiger Zusammensetzung und Kettenlänge kovalent oder durch Chemi- bzw. Physisorption angebunden sind und die modifizierten Nukleinsäure-Oligomere kovalent an diese Molekülteile angebunden sind.
- 39.) Modifizierte leitfähige Oberfläche nach Anspruch 38, wobei die kürzeste durchgehende Verbindung zwischen der leitfähigen Oberfläche und dem Nukleinsäure-Oligomer ein verzweigtes oder unverzweigtes Molekülteil mit einer Kettenlänge von 1 20 Atomen, insbesondere von 1 12 Atomen, ist.
- 40.) Modifizierte leitfähige Oberfläche nach den Ansprüchen 38 oder 39, wobei der verzweigte oder unverzweigte Molekülteil alternativ an eine Phosphorsäure-, Carbonsäure-, eine Amin- oder eine Zucker-Gruppe, insbesondere eine Zucker-Hydroxy-Gruppe, des Nukleinsäure-Oligomer-Rückgrats oder eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer modifizierten Base des Nukleinsäure-Oligomers angebunden ist.
- 41.) Modifizierte leitfähige Oberfläche nach Anspruch 40, wobei der verzweigte oder unverzweigte Molekülteil an eine Phosphorsäure-, Zucker-Hydroxy-, Carbonsäure- oder Amin-Gruppe am Ende des Nukleinsäure-Oligomer-Rückgrats bzw. eine Thiol-, Hydroxy-, Carbonsäure- oder Amin-Gruppe einer endständigen, modifizierten Base gebunden ist.
- 42.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 41, dadurch gekennzeichnet, daß jeweils überwiegend eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.

43.) Modifizierte leitfähige Oberfläche nach einem der Ansprüche 29 - 41, dadurch gekennzeichnet, daß jeweils ausschließlich eine Art von modifizierten Nukleinsäure-Oligomeren in einem räumlich begrenzten Bereich der leitfähigen Oberfläche angebunden ist.

- 44.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 29 43 definiert, dadurch gekennzeichnet, daß ein oder mehrere Arten von modifizierten Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht werden.
- 45.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche wie in den Ansprüchen 29 43 definiert, dadurch gekennzeichnet, daß ein oder mehrere Arten von Nukleinsäure-Oligomeren auf eine leitfähige Oberfläche aufgebracht werden und anschließend eine Modifikation der Nukleinsäure-Oligomere durch ein Verfahren gemäß den Ansprüchen 21 28 durchgeführt wird.
- 46.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach Anspruch 44 oder 45, wobei die Nukleinsäure-Oligomere oder die modifizierten Nukleinsäure-Oligomere mit dem dazu jeweils komplementären Nukleinsäure-Oligomerstrang hybridisiert werden und in Form des Doppelstranghybrids auf die leitfähige Oberfläche aufgebracht werden.
- 47.) Verfahren zur Herstellung einer modifizierten leitfähigen Oberfläche nach den Ansprüchen 44 oder 45, wobei das Nukleinsäure-Oligomer oder das modifizierte Nukleinsäure-Oligomer in Gegenwart von weiteren chemischen Verbindungen, die ebenfalls an die leitfähige Oberfläche angebunden werden, auf die leitfähige Oberfläche aufgebracht wird.
- 48.) Verfahren zur elektrochemischen Detektion von Oligomer-Hybridisierungsereignissen, dadurch gekennzeichnet, daß eine oder mehrere modifizierte leitfähige Oberflächen, wie in den Ansprüchen 29 - 43 definiert, mit Nukleinsäure-Oligomeren in Kontakt gebracht werden und anschließend eine Detektion der elektrischen Kommunikation zwischen der redoxaktiven Einheit und der jeweiligen leitfähigen Oberfläche erfolgt.
- 49.) Verfahren nach Anspruch 48, wobei die Detektion cyclovoltametrisch, amperometrisch oder durch Leitfähigkeitsmessung erfolgt.

50.) Verfahren zur elektrochemischen Detektion nach den Ansprüchen 48 oder 49, dadurch gekennzeichnet, daß die elektrochemische Detektion durch photoinduzierte Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktive Einheit gestartet wird.

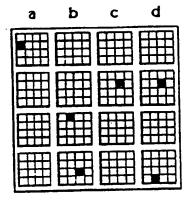
- 51.) Verfahren nach Anspruch 50, wobei die Lichteinstrahlung zur photoinduzierten Ladungstrennung in der über ein Nukleinsäure-Oligomer an die leitfähige Oberfläche angebundenen photoinduzierbar redoxaktive Einheit auf einen Bereich der leitfähigen Oberfläche mit einer oder mehreren modifizierten Nukleinsäure-Oligomeren-Arten begrenzt wird.
- 52.) Verfahren nach einem der Ansprüche 50 oder 51, wobei das nach Einstrahlen von Licht bestimmter oder beliebiger Wellenlänge entstandene oxidierte Elektron-Donor-Molekül oder das entstandene reduzierte Elektron-Akzeptor-Molekül der photoinduzierbar redoxaktiven Einheit durch eine geeignete, freie, nicht an das Nukleinsäure-Oligomer gebundene über mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz, re-reduziert bzw. reoxidiert, also jeweils in seinen ursprünglichen vor der Lichteinstrahlung vorhandenen Zustand, zurückversetzt wird.
- 53.) Verfahren zur elektrochemischen Detektion nach den Ansprüchen 48 oder 49, dadurch gekennzeichnet, daß die elektrochemische Detektion durch eine freie redoxaktive Substanz, durch die ein chemisch induzierter Ladungstransfer auf die redoxaktive Einheit stattfindet, ermöglicht wird.
- 54.) Verfahren nach Anspruch 52 oder 53, wobei die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz bei einem Potential selektiv oxidierbar und reduzierbar ist, wobei der Bedingung 2,0 V 2,0 V, gemessen gegen Normalwasserstoffelektrode, genügt.
- 55.) Verfahren nach einem der Ansprüche 52 54, wobei die freie, nicht an ein Nukleinsäure-Oligomer gebundene, aber mit dem Nukleinsäure-Oligomer in Kontakt stehende, redoxaktive Substanz ein freies Chinon, ein freier Hexacyanoferrat(II)-Komplex, ein freies Natriumascorbat, ein freier Ru(II)hexamin-Komplex oder ein freies redoxaktives Protein, insbesondere ein freies Cytochrom, ist.

Fig. 1

DNA-Fragment

5'-...AGTCCCTTGGCTC...-3'

Sequanzbestimmung durch Mustererkennung



3'-TCAGGGAA-5' (3'-CAGGGAAC-5' 3'-AGGGAACC-5' 3'-GGAACCG-5' 3'-GGAACCGA-5' 3'-GAACCGAG-5'

Oktamer 1
Oktamer 2
Oktamer 3
Oktamer 4
Oktamer 5
Oktamer 6



3'-TCAGGGAACCGAG-5' zusammengesetzte

zusammengesetzte Komplementärsequenz



5'-...AGTCCCTTGGCTC...-3' deduzierte Sequenz des DNA-Fragments

Fig. 2

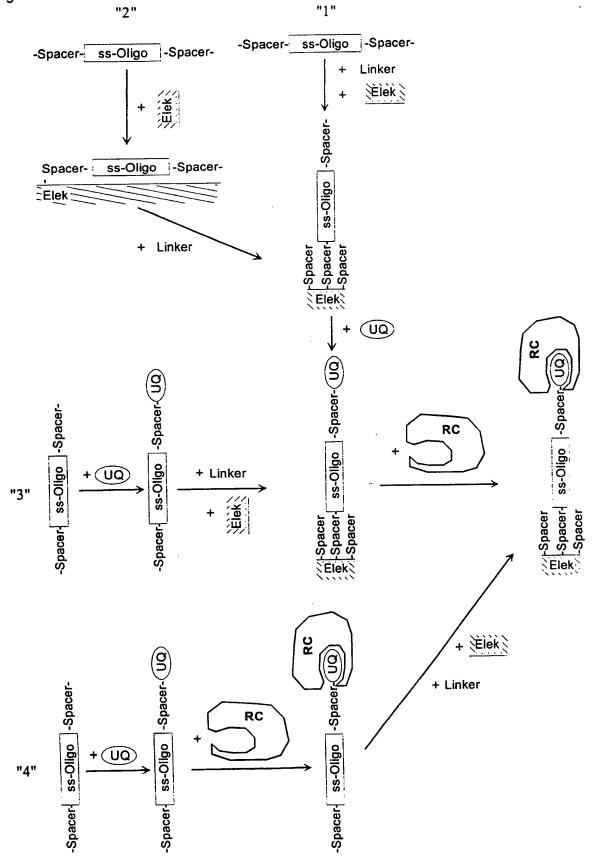
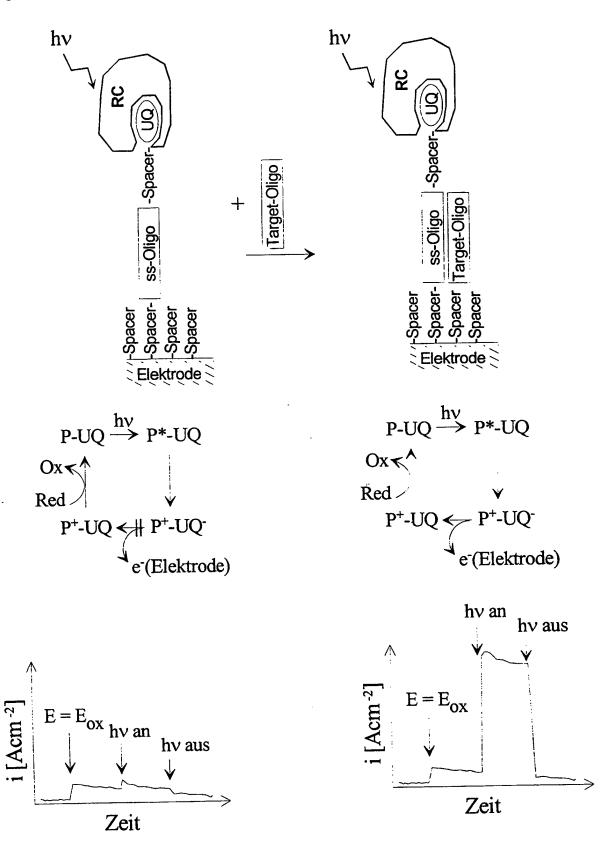
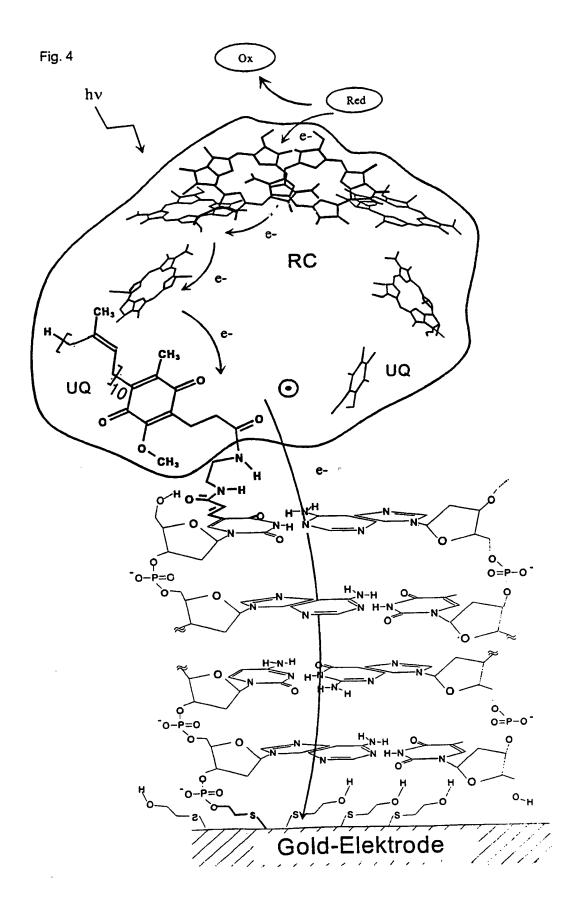
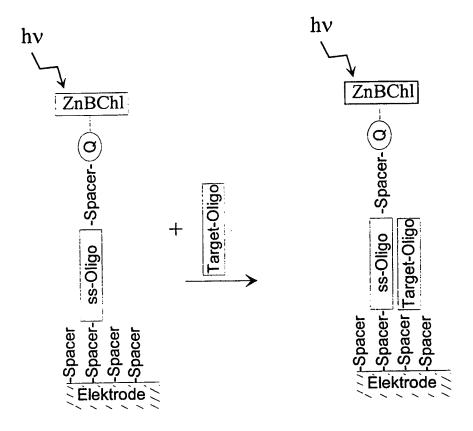
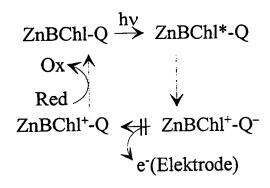


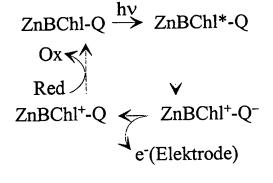
Fig. 3

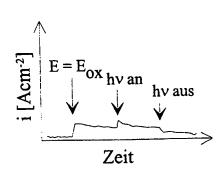












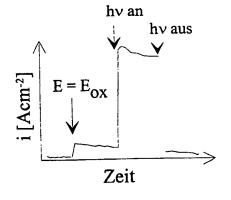
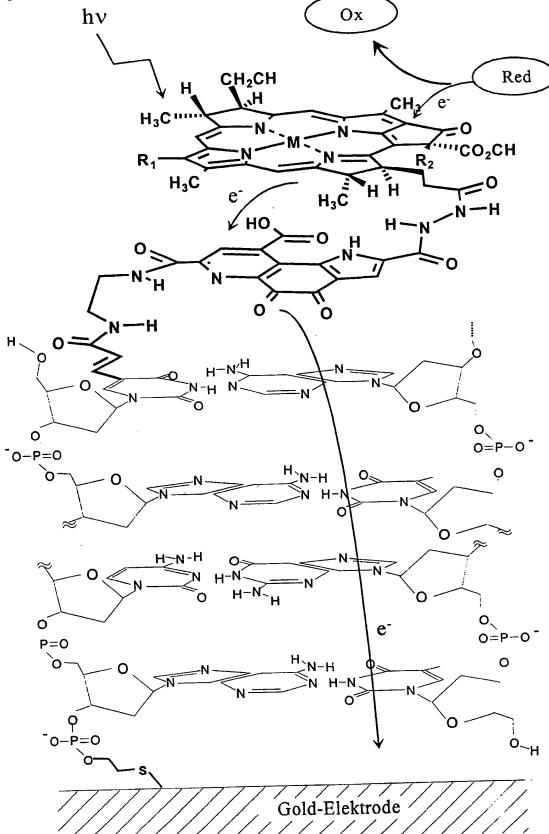


Fig. 5

Fig. 6



LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

	• 10 t	ES	Spanien	LS	Lesotho	SI	Slowenien
L	Albanien	FI	Finnland	LT	Litauen	SK	Slowakci
M	Armenien	FR	Frankreich	LU	Luxemburg	SN	Senegal
T	Osterreich	GA.	Gabun	LV	Lettland	SZ	Swasiland
U	Australien	GB	Vereinigtes Königreich	MC	Monaco	TD	Tschad
LΖ	Aserbaidschan	GE	Georgien	MD	Republik Moldau	TG	Togo
3A	Bosnien-Herzegowina		Ghana	MG	Madagaskar	TJ	Tadschikistan
B	Barbados	GH GN	Guinea	MK	Die ehemalige jugoslawische	TM	Turkmenistan
E	Belgien	_	Griechenland	*****	Republik Mazedonien	TR	Türkei
3F	Burkina Faso	GR		ML	Mali	TT	Trinidad und Tobago
BG	Bulgarien	HU	Ungam	MN	Mongolei	UA	Ukraine
gj.	Benin	IE	Irland	MR	Mauretanica	UG	Uganda
3	Brasilien	IL	Israel	MW	Malawi	US	Vereinigte Staaten von
	Belarus	IS	Island	MX	Mexiko		Amerika
	Kanada	IT	Italien			UZ	Usbekistan
	Zentralafrikanische Republik	JP	Japan	NE	Niger Niederlande	VN	Vietnam
	Kongo	KE	Kenia	NL		ΥÜ	Jugoslawien
	chweiz	KG	Kirgisistan	NO	Norwegen	zw	Zimbabwe
	e d'Ivoire	KP	Demokratische Volksrepublik	NZ	Neusceland	211	Zillioaowc
	ะกาก		Korea	PL	Polen		
		KR	Republik Korea	PT	Portugal		
		ΚZ	Kasachstan	RO	Rumanien		
	he Republik	LC	St. Lucia	RU	Russische Föderation		
	•	LI	Liechtenstein	SD	Sudan		
		LK	Sri Lanka	SE	Schweden		
		LR	Liberia	SG	Singapur		

INTERNATIONALE TO TCHERCHENBERICHT

tionales Aktenzeichen

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES IPK 7 C12Q1/68 C07H21/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole) $IPK \ 7 \quad C12Q \quad C07H$

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. ALS WE	SENTLICH ANGESEHENE UNTERLAGEN		
Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich untr - Angabe	e der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	US 5 770 369 A (FRASER SCOTT E E 23. Juni 1998 (1998-06-23) das ganze Dokument	T AL)	1–55
X	WO 98 20162 A (GOZIN MICHAEL ;YU (US); KAYYEM JON F (US); CLINICAL 14. Mai 1998 (1998-05-14) das ganze Dokument		1-5, 21-23, 29,30
X	US 5 622 946 A (HARRIMAN ANTHONY 22. April 1997 (1997-04-22) Beispiel 4	M ET AL)	1-5, 21-23
X	DE 42 16 696 A (DEUTSCHE AEROSPAC 28. Oktober 1993 (1993-10-28) das ganze Dokument 	E) -/	1-5
	l ere Veröffentlichungen sind der Fortsetzung von Feld C zu ehmen	X Siehe Anhang Patentfamilie	-
"A" Veröffe aber r "E" älteres Anme "L" Veröffe scheir ander soll or ausge "O" Veröffe eine E "P" Veröffe dem b	entlichung, die sich auf eine mündliche Offenbarung, benutzung, eine Ausstellung oder andere Maßnahmen bezieht mitlichung, die vor dem internationalen Anmeldedatum, aber nach beanspruchten Prioritätsdatum veröffentlicht worden ist Abschlusses der internationalen Recherche	kann nicht als auf erfinderischer Tätigh werden, wenn die Veröffentlichung mit Veröffentlichungen dieser Kategorie in diese Verbindung für einen Fachmann "&" Veröffentlichung, die Mitglied derselber Absendedatum des internationalen Re	t worden ist und mit der rzum Verständnis des der oder der ihr zugrundeliegenden utung; die beanspruchte Erfindungchung nicht als neu oder auf achtet werden utung; die beanspruchte Erfindung eit beruhend betrachtet einer oder mehreren anderen Verbindung gebracht wird und naheliegend ist natentamilie ist
6	. September 2000	13/09/2000	

2

Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016

Reuter, U

INTERNATIONALE TCHERCHENBERICHT

tionales Aktenzeichen
PUT/EP 00/00084

Kategorie®	ung) ALS WESENTLICH ANGESEHENE UNTERLAGEN Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	US 5 312 527 A (MIKKELSEN SUSAN R ET AL) 17. Mai 1994 (1994-05-17) das ganze Dokument	1-55
Α	KATZ E ET AL: "ELECTROCHEMICAL STUDY OF PYRROLOQUINOLINE QUINONE COVALENTLY IMMOBILIZED AS A MONOLAYER ONTO A CYSTAMINE-MODIFIED GOLD ELECTRODE" JOURNAL OF ELECTROANALYTICAL CHEMISTRY AND INTERFACIAL ELECTROCHEMISTRY, NL, ELSEVIER, AMSTERDAM, Bd. 367, 1994, Seiten 59-70, XP000672483 ISSN: 0022-0728 das ganze Dokument	1-55
A	WO 98 31839 A (HARVARD COLLEGE) 23. Juli 1998 (1998-07-23) das ganze Dokument	1-55
A	EP 0 831 327 A (BOEHRINGER MANNHEIM GMBH) 25. März 1998 (1998-03-25) das ganze Dokument	1-55
Ρ,Χ	WO 99 51778 A (BARTON JACQUELINE ;HILL MICHAEL (US); KELLEY SHANA (US); CALIFORNI) 14. Oktober 1999 (1999-10-14) das ganze Dokument	1-5, 21-23, 29,30
Ε	WO 00 31101 A (HARTWICH GERHARD ; HELLER ADAM (US)) 2. Juni 2000 (2000-06-02) das ganze Dokument	1-55

INTEF TIONAL SEARCH REPORT

Information on patent family members

tional Application No
PUT/EP 00/00084

Patent document cited in search report		Publication date		atent family nember(s)	Publication date
· · · · · · · · · · · · · · · · · · ·				5824473 A	20-10-1998
US 577036 9	Α	23-06-1998	US US	5591578 A	07-01-1997
			US US	6071699 A	06-06-2000
				5952172 A	14-09-1999
			US		30-12-1996
			AU	6166296 A	21-10-1998
			EP	0871642 A	19-12-1996
			WO	9640712 A	25-03-1999
			AU	703329 B	
			AU	1215295 A	27-06-1995
			CA	2178618 A	15-06-1995
			EP	0733058 A	25-09-1996
			JP	9506510 T	30-06-1997
			WO	9515971 A	15-06-1995
			US	5780234 A	14-07-1998
			US	5705348 A	06-01-1998
			US	6087100 A	11-07-2000
WO 9820162	 А	14-05-1998	US	6096273 A	01-08-2000
MO SOCOTOR	^	1, 00 1550	ÜS	6090933 A	18-07-2000
			ĂŬ	5196798 A	29-05-1998
			EP	0939762 A	08-09-1999
US 5622946	 A	22-04-1997	 US	5457183 A	10-10-1995
U3 3022940	М	77 04-1331	US	6072038 A	06-06-2000
			US	5583220 A	10-12-1996
			US	5580543 A	03-12-1996
			US US	5587371 A	24-12-1996
					27-05-1997
			US	5632970 A	01-09-1998
			US	5801229 A	19-10-1999
			US	5969111 A	
			US	5888997 A	30-03-1999
			US	6069140 A	30-05-2000
			AU	683316 B	06-11-1997
			AU	8075694 A	04-05-1995
			CA	2173319 A	20-04-1995
			EP	0724457 A	07-08-1996
			JP	9508616 T	02-09-1997
			NO	961436 A	11-06-1996
			SG	48392 A	17-04-1998
			WO	9510307 A	20-04-1995
			US	5599923 A	04-02-1997
DE 4216696	Α	28-10-1993	W0	9321530 A	28-10-1993
US 5312527	Α	17-05-1994	NONE		
WO 9831839	Α	23-07-1998	AU		07-08-1998
			EP	0981643 A	01-03-2000
FP 0831327		25-03-1998	DE	19639169 A	02-04-1998
_, ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	. ,		CA	2216105 A	
			JP		19-05-1998
			ÜS	6057120 A	02-05-2000
 WO 9951778	Α	14-10-1999 	AU	3550699 A	25-10-1999